

**REPRODUCTIVE HEALTH ASSESSMENT OF  
FATHEAD MINNOW (*PIMEPHALES PROMELAS*)  
POPULATIONS INHABITING AN EFFLUENT  
DOMINATED STREAM, WASCANA CREEK, SK**

A Thesis Submitted to the College Of  
Graduate and Postdoctoral Studies  
In Partial Fulfillment of the Requirements  
For the Degree of Master of Science  
In the Toxicology Program of  
University of Saskatchewan  
Saskatoon, Saskatchewan, Canada

Sara Hanson

## **PERMISSION TO USE**

In presenting this thesis in partial fulfillment of the requirements for a postgraduate degree from the University of Saskatchewan, I agree that the Libraries of the University may make it freely available for inspection. I further agree that permission for copying of this thesis in any manner, in whole or in part, for scholarly purpose may be granted by the professors who supervised my thesis work or, in their absence, by the Head of the Department or the Dean of the College in which my thesis work was done. It is understood that any copying or publication or use of this thesis or parts thereof for financial gain shall not be allowed without my written permission. It is also understood that due recognition shall be given to me and the University of Saskatchewan in any scholarly use which may be made of any material in my thesis.

Requests for permission to copy or to make other use of material in this thesis in whole or parts should be addressed to:

Dean  
College of Graduate and Postdoctoral Studies  
University of Saskatchewan  
116 Thorvaldson Building, 110 Science Place  
Saskatoon, Saskatchewan S7N 5C9, Canada

Chair of the Toxicology Graduate Program  
Toxicology Centre  
University of Saskatchewan  
44 Campus Drive  
Saskatoon, Saskatchewan, S7N 5B, Canada

## ABSTRACT

Concerns have been raised regarding the release of municipal wastewater effluents (MWWEs) into aquatic systems in recent decades due to their potential adverse effects on resident aquatic organisms. MWWEs contain complex mixtures of contaminants, and many of which are considered to have endocrine disrupting properties that may alter growth, development and reproduction of exposed organisms. Waterbodies in the southern Canadian Prairies, a semi-arid region, may be at particular risk to the exposure with contaminants released within MWWEs, including endocrine disrupting chemicals (EDCs), due to the uniqueness of prairie surface water systems. For example, during low flow periods small prairie streams such as Wascana Creek, SK downstream of the City of Regina's outdated lagoon-based treatment facility can contain up to 100% treated effluent. Therefore, the objective of this study was to evaluate the potential impacts of MWWEs on reproductive health and endocrine homeostasis of wild fathead minnow (*Pimephales promelas*; *FHMs*) populations downstream of the City of Regina's WWTP in Wascana Creek, Saskatchewan, Canada. Specifically, field studies were conducted on spawning FHMs during the summers of 2014 and 2015 to assess responses in terms of overall health (condition factor, ovosomatic indices) and reproduction (secondary sexual characteristics, gonad histopathology), and to evaluate molecular biomarkers (plasma hormone analysis, gene expression analysis) to determine any potential underlying mechanisms. Additionally, chemical analysis was done on surface water samples from both up- and down-stream sites to identify potential causative agents for any observed biological effects. FHMs downstream of the effluent fallout had lower gonadosomatic indices and significantly greater hepatosomatic indices compared to upstream populations. In both male and female FHMs significantly greater occurrence and severity of gonadal degradation and delayed maturation were observed in downstream fish compared to

upstream fish; however, no indications of exposure to estrogenic compounds such as occurrence of testicular oocytes were observed. Furthermore, exposed males displayed lower scores of secondary sexual characteristics. While no significant differences were found in plasma hormone concentrations between fish populations up- and down-stream of the City of Regina's wastewater treatment plant, trends of increased estradiol and decreased 11-ketotestosterone in females and males, respectively, were detected between years and sampling sites. Key biomarkers of estrogenic exposure, such as induction of vitellogenin (VTG), were not observed in exposed male FHMs. Lowered expression of estrogen responsive genes ( $ER\alpha$ ,  $ER\beta$ , VTG) in populations downstream suggest that estrogenic compounds likely did not cause the effects observed in the parallel study. In exposed fish downstream there was evidence of down-regulation of key steroidogenic genes, CYP19 $\beta$  and StAR, coupled with lowered FSHR and LHR expression, suggesting that steroidogenic capacity in the gonads of fish exposed to the MWWEs had been reduced. Overall, delays in maturation and histological alterations evident in populations downstream were supported by the general suppressive molecular responses. The general inhibition of reproductive functions of fish at RDS are in accordance with two parallel studies with lab exposed fish showing reduced fecundity in addition to similar delays in maturation and predominant antagonistic potentials detected *in vitro* by ER and AR receptor assays. Taking into account the overall lack of estrogenic effects across the *in vivo* studies (ie. Lack of VTG induction and no occurrence of intersex) and low estrogenic potential of the effluents entering RDS reported by the *in vitro* study, it is likely that the effects observed were not due to estrogenicity in the effluents. This is also in accordance with chemical analysis that revealed greater concentrations of compounds with the ability to act as ER and AR antagonists, while there were low, or no presence of chemicals previously shown to agonistically interact with these receptors. With

continuous exposure to a diverse number of stressors including high nutrient and ammonia levels and presence of a variety of PPCPs and other contaminants, Wascana Creek should be considered as an ecosystem at risk with regard to effects from MWWs on resident fish populations. While this study was regional in nature, due to similarities, inferences can likely be made among different semi-arid regions around the world in regards to the ecological impacts of MWWs.

## **ACKNOWLEDGEMENTS**

I would like to thank my graduate supervisor Dr. Markus Hecker for his continued guidance, support and mentorship throughout my Masters. I would like to extend my thanks to the members of my graduate committee, Dr. Steve Wiseman, Dr. Natacha Hogan and Dr. Lynn Weber for their comments, support and advice. I would also like to thank my external examiner, Dr. Vicki Marlatt.

I would like to thank Ashley Moate, Bryanna Eisner, Danielle Gagnon, Shawn Beitel and Jon Doering for their help with lab and field assistance. Also thank you to Myles Stocki for his assistance with stable isotope analysis and to the City of Regina Wastewater Treatment Plant for their cooperation.

I would like to acknowledge my funding sources for this project including Dr. Markus Hecker's Canadian Water Network Grant, the Canada Research Chairs Program and the Toxicology Devolved scholarships.

Most importantly, I would like to express my deepest gratitude to my family and friends for their overwhelming encouragement, support and understanding throughout this thesis. Finally, a special thank you to Drew Kurtz for his love, patience and support throughout the duration of my Masters.

# TABLE OF CONTENTS

PERMISSION TO USE .....	i
ABSTRACT .....	ii
ACKNOWLEDGEMENTS .....	v
LIST OF TABLES .....	viii
LIST OF FIGURES .....	ix
LIST OF ABBREVIATIONS .....	xi
PREFACE .....	xv
<b>Chapter 1 : GENERAL INTRODUCTION .....</b>	<b>1</b>
1.1 Endocrine Disrupting Compounds .....	1
1.1.1 Mechanisms of EDC Action .....	2
1.2 Sources of EDCs in the Aquatic Environment .....	3
1.2.1 Municipal Wastewater Effluents .....	3
1.2.2 Levels of Treatment of Municipal Wastewater Effluent in Canada.....	5
1.2.3 MWWE as an Endocrine Disruptor .....	6
1.3 WWTPs, MWWEs and Receiving Waterbodies in Saskatchewan.....	8
1.3.1 Surface Water Issues in Saskatchewan .....	8
1.3.2 Wascana Creek, Regina, Saskatchewan, Canada.....	9
1.4 Fathead Minnow as Test Species for EDCs .....	10
1.5 Biological Endpoints of Endocrine Disruption in the Fathead Minnow .....	11
1.5.1 Hypothalamic-Pituitary-Gonadal-Liver Axis in Fish.....	12
1.5.2 Histopathology of Gonads .....	14
1.5.3 Secondary Sexual Characteristics .....	18
1.5.4 Organosomatic Indices – GSI, HSI, K.....	19
1.6 Objectives and Hypotheses.....	20
<b>Chapter 2 : HEALTH STATUS OF FATHEAD MINNOW (<i>PIMEPHALES PROMELAS</i>)</b>	
<b>POPULATIONS IN A MUNICIPAL WASTE WATER EFFLUENT-DOMINATED</b>	
<b>STREAM IN THE CANADIAN PRAIRIES, WASCANA CREEK, SK. ....</b>	<b>23</b>
PREFACE .....	24
2.1 Abstract.....	26
2.2 Introduction .....	27
2.3 Materials and Methods .....	30
2.3.1 Site Location and Field Collections .....	30
2.3.2 Tissue Sampling and Morphometrics .....	33
2.3.3 Histological Analysis .....	33
2.3.4 Stable Isotope Analysis.....	35
2.3.5 Chemical Analysis .....	35
2.3.6 Statistical Analysis.....	37
2.4 Results .....	37
2.4.1 Fish Health .....	40
2.4.2 Histopathology .....	42

2.4.3 Stable isotope analysis .....	46
2.4.4 Chemical analysis .....	48
2.5 Discussion.....	50
2.6 Conclusions .....	60
<b>Chapter 3 : MOLECULAR BIOMARKERS AS MECHANISTIC EVIDENCE OF MUNICIPAL WASTEWATER EFFLUENT EFFECTS ON WILD FATHEAD MINNOW (<i>PIMEPHALES PROMELAS</i>) POPULATIONS IN AN EFFLUENT DOMINATED STREAM, WASCANA CREEK, SK, CANADA.....</b>	<b>62</b>
PREFACE .....	63
3.1 Abstract.....	65
3.2 Introduction .....	66
3.3 Materials and Methods .....	70
3.3.1 Field Sampling and tissue collection .....	70
3.3.2 Plasma hormone analysis .....	72
3.3.3 Quantitative real-time polymerase chain reaction.....	73
3.3.4 Statistical Analysis.....	75
3.4 Results .....	75
3.5 Discussion.....	82
3.6 Conclusions .....	88
<b>Chapter 4 : GENERAL DISCUSSION.....</b>	<b>90</b>
4.1 Introduction.....	90
4.2 Summary of Findings.....	91
4.3 AIME Project Findings .....	97
4.4 Future Work .....	100
4.5 Conclusions.....	101
REFERENCES .....	103
Appendix A: Supplemental Materials.....	112
Appendix B: Histological Staining .....	113
Appendix C: Histological Grading and Staging Scale.....	114



## LIST OF TABLES

<b>Table 2.1.</b> Average, maximum and minimum concentrations of ammonia nitrogen (mg/L), unionized ammonia at 15±1°C (mg/L), total phosphorus (mg/L) and total nitrogen (mg/L) in final effluents at the City of Regina’s WWTP in July for both 2014 and 2015.....	39
<b>Table 2.2.</b> Mean (± SE) (n) length, body weight, condition factor (k), hepatosomatic index (HSI), and gonadosomatic index (GSI) of Fathead Minnows collected in Wascana Creek, SK. Differences among sites (p<0.05) are denoted by different lowercase letters.....	41
<b>Table 2.3.</b> Summary of analytical results of in-stream water samples from RUS and RDS in 2014 for 22 organic wastewater contaminants. Chemical concentrations are shown in ng/L. Concentrations below the method detection limit are denoted by <MDL.....	49
<b>Table 3.1.</b> Nucleotide sequence, efficiencies and GenBank accession numbers of primer pairs used in qPCR for brain, gonad and liver tissues. ....	74
<b>Table 4.1.</b> Summary of responses for whole organism, tissue, steroid hormones and gene expression for female (F) and male (M) fathead minnows collected downstream of the effluent fallout in Wascana Creek, SK (RDS). (↑) statistically significant increase, (↓) statistically significant decrease, (-) difference from control group is not statistically detectable. ....	96
<b>Table 4.2.</b> Summary of responses for entire AIME project with in vivo (wild fish studies, this thesis; 21 reproductive assay, Steeves, 2018) endpoints for female (F) and male (M) fathead minnows and in vitro bioassay potentials (Bagatim, 2019). (↑) statistically significant increase, (↓) statistically significant decrease, and (--) not applicable. ....	99

## LIST OF FIGURES

**Figure 1.1.** Sample gonadal micrographs from fathead minnow. (A) Reference ovary with small, dark, primary-stage oocytes situated between cortical alveolar-stage oocytes and large vitellogenic oocytes. (B) EE2 exposed ovary displaying atretic follicle (arrow). (C) Reference testis. (D) EE2 exposed testes demonstrating testicular oocytes: arrows indicate primary-stage oocytes among the remnants of testicular tissue (from Kidd et al., 2007)..... 16

**Figure 2.1.** Site locations for sampling of Fathead Minnows in Wascana Creek. RUS (Regina Upstream Site); RDS (Regina Downstream Site); MWWE (Municipal wastewater effluent)..... 32

**Figure 2.2.** Average severity grading's ( $\pm$ SEM) for associated histopathological primary criteria of males (A and B), and females (D) at sampling locations upstream (RUS) and downstream (RDS) of the City of Regina in 2014. Average maturity grade of females (C). \* =  $p < 0.05$  and \*\* =  $p < 0.001$ . ..... 43

**Figure 2.3.** Stained gonadal tissues from adult male and female adult FHMs collected in Wascana Creek upstream (left; RUS) and downstream (right; RDS) of the City of Regina in 2014. Adult male testes (1) containing spermatogonia (SG), spermatocytes (SC), spermatids (ST), and spermatozoa (SZ). RDS male (1b) showing moderate (grade 3) increase in proportion of spermatogonia (SG) compared to RUS male (1a). Male upstream (2a) displaying non-remarkable degeneration, with RDS male (2b) showing a mild (grade 2) increase in testicular degeneration with losses of cell architecture, apoptotic germ cell formation and cell shrinkage and fragmentation. Ovarian tissue showing non-remarkable atresia in females upstream (3a) and a moderate (grade 3) increase in oocyte atresia in females downstream (3b). Asterisks (\*) indicate the relatively few non-atretic oocytes in the exposed female tissue. (4) Mid-development (stage 2) was observed in upstream females (4a) with vitellogenic (V) and cortical alveolar (CA) follicles, while undeveloped (stage 0) was observed in downstream females (4b) with the tissue consisting of entirely primary (P) follicles (paraffin, H&E)..... 45

**Figure 2.4.** Stable isotope bi-plot showing nitrogen  $\delta^{15}\text{N}$  and carbon  $\delta^{13}\text{C}$  values (mean  $\pm$  SEM) for sites RUS and RDS in 2014 and 2015. Statistically significant differences between sites are denoted by lower case letters for  $\delta^{15}\text{N}$  and uppercase letters for  $\delta^{13}\text{C}$ ..... 47

**Figure 3.1.** Site locations for sampling of Fathead Minnows in Wascana Creek. RUS (Regina Upstream Site); RDS (Regina Downstream Site); MWWE (Municipal wastewater effluent)..... 71

**Figure 3.2.** Mean ( $\pm$  SEM) concentrations of plasma hormones (A)  $17\beta$ -estradiol (E2) in female, and (B) 11-ketotestosterone (11-KT) in male fathead minnows collected upstream (RUS) and downstream (RDS) of the effluent fallout in Wascana Creek, SK in 2014 (solid bars) and 2015 (dashed bars). Different letters indicate significant difference in the concentrations of plasma hormone between sites ( $p \leq 0.05$ ). ..... 76

**Figure 3.3.** Abundances of transcripts of genes involved in sex steroid hormone synthesis and signaling in female fathead minnows upstream (RUS; dark) and downstream (RDS; light) of the effluent fallout in Wascana Creek, SK in 2014 (left; RUS:  $n = 17$ , RDS:  $n = 10$ ) and 2015 (right; RUS:  $n = 16$ , RDS:  $n = 26$ ). (A) brain, (B), gonad, (C) liver. Data are reported as the mean  $\pm$  SEM. Statistical significance denoted by \* =  $p \leq 0.05$ . ..... 78

**Figure 3.4.** Abundances of transcripts of genes involved in sex steroid hormone synthesis and signaling in male fathead minnows upstream (RUS; dark) and downstream (RDS; light) of the effluent fallout in Wascana Creek, SK in 2014 (left; RUS:  $n = 10$ , RDS:  $n = 24$ ) and 2015 (right; RUS:  $n = 6$ , RDS:  $n = 4$ ). (A) brain, (B), gonad, (C) liver. Data are reported as the mean  $\pm$  SEM. Statistical significance denoted by \* =  $p \leq 0.05$ . ..... 81

## LIST OF ABBREVIATIONS

(anti-) – refers to both an anti-androgen/androgen and anti-estrogen/estrogen

°C – degree Celsius

µg/L – microgram per litre

µL – microliter

µm – micrometre

δ<sup>13</sup>C – carbon stable isotope signature

δ<sup>15</sup>N – nitrogen stable isotope signature

11-KT – 11-ketotestosterone

17βHSD – 17β-hydroxysteroid dehydrogenase

ANOVA – analysis of variance

AR – androgen receptor

BFR – brominated flame retardant

CA – cortical alveolar follicle

cDNA – complimentary deoxyribonucleic acid

CYP19α – aromatase (gonad)

CYP19β – aromatase (brain)

DNA – deoxyribonucleic acid

DO – dissolved oxygen

EC – emerging contaminant

EDC – endocrine disrupting compound

E1 – estrone

E2 – 17β-estradiol

EE2 - 17α-ethinylestradiol

ELISA – enzyme-linked immunosorbent assay

ER – estrogen receptor

ERα – estrogen receptor alpha

ERβ – estrogen receptor beta

FHM – fathead minnow  
FSH – follicle stimulating hormone  
FSHR – follicle stimulating hormone receptor  
g – gram  
GnRH – gonadotropin releasing hormone  
GSI – gonadosomatic index  
H & E – hematoxylin-2 and eosin-y  
HPGL – hypothalamus-pituitary-gonadal-liver axis  
HSI – hepatosomatic index  
K – condition factor  
km – kilometre  
kV – kilovolt  
L – litre  
L/h – litre per hour  
LH – luteinizing hormone  
LHR – luteinizing hormone receptor  
LOQ – limit of quantification  
 $\text{m}^3/\text{s}$  – cubic metre per second  
MDL – method detection limit  
min – minute  
mg/L – milligram per litre  
mL – millilitre  
mL/min – millilitre per minute  
ML/d – million litre per day  
Mm – millilitre  
mRNA – messenger ribonucleic acid  
ms – millisecond  
MWWE – municipal wastewater effluent

m/z – mass-to-charge ratio  
n = sample size  
ng/L – nanogram per litre  
NP – 4-nonylphenol  
OSI – organosomatic index  
P – primary follicle  
PAH – polycyclic aromatic hydrocarbons  
pg/mL – pictogram per millilitre  
POP – persistent organic pollutants  
PPCP – pharmaceuticals and personal care products  
qPCR – quantitative polymerase chain reaction  
QSAR - quantitative structure activity relationships  
RDS – Regina Downstream Site  
RNA – ribonucleic acid  
rpm – revolutions per minute  
RUS – Regina Upstream Site  
s - second  
S.E.M – standard error of the mean  
SC – spermatocytes  
SG – spermatogonia  
ST – spermatids  
StAR – steroidogenic acute regulatory protein  
SZ – spermatozoa  
T – testosterone  
UV – ultraviolet  
V – vitellogenic  
VTG – vitellogenin  
VZ - vinclozolin

WWTP – wastewater treatment plant

ZR – zona radiata protein

## PREFACE

This thesis is written in manuscript style format. Chapter 1 of this thesis is a general introduction and literature review. Chapters 2 and 3 are organized as manuscripts for publication in peer-reviewed scientific journals. Chapter 4 is a general discussion with overall conclusions from the findings in Chapters 2 and 3. Therefore, there may be some repetition between the introduction, materials and methods, results and discussions sections in each chapter. Chapter 2 is being prepared for submission to *Environmental Toxicology and Chemistry*, and Chapter 3 is being prepared for submission to *Aquatic Toxicology*.



# **Chapter 1 : GENERAL INTRODUCTION**

## **1.1 Endocrine Disrupting Compounds**

The idea that exposure to xenobiotic chemicals could inappropriately alter the endocrine system, therefore causing negative effects in wildlife and humans, dates back some 50 years, with a considerable amount of research having been conducted on endocrine disrupting compounds (EDCs) in the past three decades (WHO 2012). The WHO definition of an endocrine disruptor is “an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism or its progeny or (sub) populations” (WHO/ICPS 2002). More specifically, EDCs can interact with the endocrine system such that they cause adverse effects on endocrine homeostasis, reproduction, development and/or behaviour (Goksøyr 2006; Campbell et al. 2006). A wide variety of compounds have been identified as EDCs including pharmaceuticals and personal care products (PPCPs), phytoestrogens, polycyclic aromatic hydrocarbons (PAHs), persistent organic pollutants (POPs) as well as other industrial and agricultural chemicals such as bisphenol-A, phthalates and certain pesticides (Purdom et al., 1994; Folmar et al., 1996; Hoffman and Oris, 2006). While endocrine effects have been widely observed in a multitude of organisms, fish are particularly at risk of being exposed to EDCs as many of these compounds are directly released into the aquatic environments through various sources resulting in intermittent or continuous exposures. In the environment, fish exposed to EDCs have shown a wide variety of endocrine effects, such as feminization of male fish, masculinization of female fish and overall disruption of normal reproduction function.

### **1.1.1 Mechanisms of EDC Action**

EDCs can cause a variety of effects through a multitude of mechanisms of action. To date, the focus has been on EDCs that mimic the action of hormones, particularly estrogens, resulting in agonistic effects on cellular processes, or alternatively EDCs that act antagonistically by binding to and blocking the receptor binding sites, therefore blocking the transcriptional activation and associated downstream processes triggered through these receptors (Arukwe, 2001). The modulation of nuclear receptor co-activators is another important mechanism of endocrine disruption and is recently receiving more attention. Nuclear receptors activate transcription through direct binding of hormone response elements to specific areas of target genes while signalling for a suite of co-activator proteins and basal transcription machinery (Tabb and Blumberg, 2006). In this process, receptor activity can be changed if expression levels of the receptors and/or co-regulator mRNAs and proteins are altered. Steady-states of nuclear receptor co-activator levels have been shown to increase with exposure to estrogenic EDCs, ultimately causing the activation of the nuclear estrogen receptors alpha and beta (ER $\alpha$  and ER $\beta$ ) to be increased in the presence of such xenobiotics (Lonard et al., 2004; Blumberg et al., 2011). Another more subtle type of endocrine disruption is the competition between steroid hormone receptors and xenobiotic receptors for transcription co-activators (Tabb and Blumberg, 2006).

In addition to effects mediated through receptors such as the estrogen receptor (ER), androgen receptor (AR) and thyroid hormone receptors, there are a number of equally important routes of endocrine disruption including modulation of steroid hormone synthesis and metabolism, receptor protein degradation, and altered DNA methylation (Mills and Chichester, 2005; Tabb and Blumberg, 2006; Bjorkblom et al., 2009; Kusk et al., 2011). Several EDCs have been shown to interfere with the synthesis, metabolism, transport, storage, secretion or degradation of hormones

(Arukwe, 2001; Goksoyr, 2006; Mills and Chichester, 2005). In particular, changes in gonadal hormone production, or steroidogenesis in general, have also been observed by a number of studies, and seem to represent a common and important mechanism of endocrine disruption (Baker 2001; Craig et al., 2011; Hecker and Giesy, 2008; Higley et al., 2010). By altering the expression of genes encoding for steroidogenic enzymes as well as the activity of those enzymes, hormones are either produced in over-abundance or are no longer produced at sufficient levels, and thus available to bind to their receptors, and therefore, can alter normal physiological functions (Sumpter et al., 2002). For example, aromatase, an enzyme critical to estrogen synthesis, has been shown to be inhibited in female fathead minnows with exposure to a variety of chemicals found in the environment including pesticides, organochlorines and organotins (Miller, 1988; Sanderson, 2006; Villeneuve et al., 2006; Villeneuve et al., 2009). Ultimately, EDCs can act through a variety of mechanisms of action, allowing their effects to be wide ranging across various species.

## **1.2 Sources of EDCs in the Aquatic Environment**

EDCs are constantly entering aquatic systems through a variety of anthropogenic practices. Sources include pulp and paper mill effluents, agricultural runoff, landfill leachates, roadway runoff and municipal waste-water effluents (MWWEs; Benotti et al., 2008; Sanderson 2006; Vos et al., 2000). MWWEs are of particular concern, as they contain complex mixtures of contaminants, many of which are considered EDCs including steroidal estrogens, biodegradation products of alkylphenols, and various PPCPs and other chemicals (Desbrow et al., 1998; Mills and Chichester, 2005; Baronti et al., 2000).

### **1.2.1 Municipal Wastewater Effluents**

Municipal wastewater effluent is a complex mixture, made up of sanitary waste collected in sewers, households as well as waste from industry, commercial establishments and other

institutions including hospitals. Depending on the level of treatment, the effluent contains a range of classic and emerging contaminants including human and other organic waste, nutrients, pathogens, microorganisms, suspended solids, PPCPs, and household and industrial chemicals that are not or are only partially removed during the treatment processes. In Canada, more than 3 trillion litres of treated effluent and 150 billion litres of untreated and undertreated wastewater is released each year into surface waters such as lakes, rivers, streams and oceans (Environment Canada 2013; CCME 2006).

Over the last two decades, increasing concerns have been raised by scientists from North America and Europe over the presence of emerging contaminants (ECs) in wastewaters, groundwater and surface waters (CCME 2006; Barber et al., 2000; Bauman et al., 1990). ECs are a wide ranging class of compounds and include PPCPs, pesticides, brominated flame retardants (BFRs), plasticizers and nanoparticles (Purdom et al., 1994; Folmar et al., 1996; Hoffman and Oris., 2006). PPCPs refer to a diverse group of chemical compounds that include analgesics, antiseptics, antibiotics, musk fragrances, sunscreen agents, natural and synthetic estrogens (EE2), as well as their metabolites (CCME 2006; Campbell et al., 2006). Thousands of these chemicals are released annually from Canadian households and have been found in low concentrations in drinking water, surface water, groundwater and MWWs in North America as well as Europe (Bauman et al., 1990; Barber et al., 2000; CCME 2006). These emerging compounds are entering the aquatic environment through leaky sewers and septic systems, as well as discharge of municipal effluents due to the inadequate removal during the treatment processes. Historically, these emerging contaminants were not thought to be widely distributed, and therefore, not considered a concern. However, many of these compounds have been found to be persistent, bioaccumulative and/or toxic at low concentrations, making them a primary concern for aquatic

systems. Additionally many of these EC's found in these systems were designed to interact with specific biological targets and elicit effects at relatively low concentrations, raising concerns of impacts on non-target aquatic organisms, particularly in regards to their endocrine disrupting capabilities (Waiser et al., 2011b).

### **1.2.2 Levels of Treatment of Municipal Wastewater Effluent in Canada**

Levels of wastewater treatment vary across Canada, ranging from no treatment or screening of solids to state-of-the-art quaternary treatment technologies. Preliminary and primary treatment involves screening and settling of solids. Secondary treatment follows with the removal of biodegradable organic matter and suspended solids using microorganisms. Sewage lagoons, a form of secondary treatment, are one of the most common biological treatment processes in Canada due to the low operational cost and simple operation. However, they are somewhat limited in their efficiency to remove constituents of concern from wastewater and greatly vary depending on their size, type and configuration of the treatment cells (i.e. anaerobic cells, facultative cells and storage cells; CCME, 2006). Additionally, efficiency of lagoon-based treatment systems may be affected by temperature and precipitation resulting in either greater loading of contaminants when efficiency is reduced in cold temperatures, or alternatively increased dilution in the lagoons following large precipitation events. Tertiary treatment is additional treatment used to remove suspended, colloidal and dissolved constituents using chemical, biological or physical processes such as sand filtration to further remove nutrients (Environment Canada, 2010). Nutrient removal methods can be incorporated into the secondary or tertiary treatment to enhance the removal of phosphorus and nitrogen. Advanced treatment facilities implicate quaternary treatment to further enhance the quality of MWW through the use of reverse osmosis, membrane filtration and activated carbon technologies. Lastly, disinfection systems are used to lower levels of indicator

microorganisms such as *E. coli* using appropriate dosages of chlorine, hypochlorite or ultraviolet (UV) radiation (CCME 2006). In general, treatment plants across Canada are not designed or built to remove trace contaminants such as EDCs and PPCPs found at very low concentrations (CCME, 2006).

### **1.2.3 MWWE as an Endocrine Disruptor**

Effluents from wastewater treatment plants (WWTPs) are released into a wide variety of receiving environments: lakes, ponds, streams, rivers, estuaries and oceans. The effluents released from wastewater systems do contain pollutants of concern, such as EDCs, since even advanced treatment systems are unable to remove all pollutants and chemicals (EC 2010).

Municipal wastewater effluents have been identified as a major cause of endocrine disrupting effects in fish in both European and North American fresh water environments (Purdom et al., 1994; Folmar et al., 1996). Particular focus has been placed on the estrogenicity of the effluents, with a vast amount of the scientific literature that has been produced focusing on effects mediated through the estrogen receptor and chemicals that interact with it. Reports from the UK showed that a large number of sewage treatment plant effluents were estrogenic to fish resulting in feminization of males in downstream areas (Purdom et al., 1994; Sumpter and Jobling, 1995), and that a number of receiving waters showed elevated estrogenic activity (Harries et al., 1996). Endocrine disrupting effects in aquatic wildlife downstream of WWTPs have not been conclusively linked to only one particular compound in the sewage discharges, but certain chemicals were hypothesized to be mainly responsible for these disruptions. Among them, the natural estrogens estrone (E1), 17 $\alpha$ -estradiol ( $\alpha$ E2) and 17 $\beta$ -estradiol ( $\beta$ E2), and the exogenous 17 $\alpha$ -ethinylestradiol (EE2), the active ingredient in oral contraceptive pills, possess the greatest estrogenic potencies (Vos et al., 2000). In addition to these steroids, alkyl-phenols such as 4-tert

octylphenol and 4-nonylphenol (NP), which are both breakdown products of detergents and surfactants, and bisphenol A, a widely used monomer for epoxy resins and polycarbonates, show estrogenic activity at four or more orders of magnitude less than  $\beta$ E2 (Kuch and Ballschmiter, 2001; Desbrow et al., 1998). Chemical analysis of caged rainbow trout in the receiving waters of MWWs revealed EE2, E1, nonylphenols and bisphenol A were present in conjugated form in the bile of the fish (Vos et al., 2000). Male fish from these experiments had significantly and highly induced plasma vitellogenin (VTG) concentrations, indicating strong estrogenic properties of the effluents of all sewage treatment works tested (Sumpter and Jobling, 1995). In addition to increased VTG in males, the presence of female germ cells in the testes, delayed gonadal maturation and altered secondary sexual characteristics have also been frequently reported (Ankley et al., 2001; Diniz et al., 2005; Hecker et al., 2002; Kidd et al., 2007; Tetreault et al., 2011). In effluents that contained anti-estrogenic compounds, female fish have been observed to have decreased levels of vitellogenin and zona radiata (Zr) proteins resulting in eggshell abnormalities, lower egg quality and reduced hatching and survival success (Arukwe and Goksoyr, 2003; Arukwe, 2001).

In addition to (anti-)estrogenic compounds, (anti-)androgenic compounds associated with MWWs may also interfere with the normal functioning of the endocrine system, resulting in reproductive disruption in exposed organisms such as fish. While the scientific literature for (anti-)androgenic effects is not as well documented as that for (anti-)estrogenic effects, many studies have reported masculinization of female fish after exposure to androgens (Arukwe, 2001; Bortone et al., 1989; Drysdale and Bortone, 1989). Pregnant female mosquitofish exposed to pulp and paper mill effluent developed an anal fin called a gonopodium, which is normally only found in males and is androgen dependent (Arukwe, 2001). Similarly, female fathead minnows exposed

to methyltestosterone in another study formed nuptial tubercles normally found only on male fathead minnows (Arukwe, 2001). Other masculinization manifestations in female fish include hermaphroditic conditions and altered behaviours (Arukwe, 2001). Exposure to anti-androgenic compounds typically shows demasculinization effects in male fish (Martinovic et al., 2007). For example, vinclozolin (VZ), an anti-androgenic chemical frequently found in the aquatic environment, has been shown to decrease the expression of secondary sexual characteristics in male fathead minnows (Martinovic et al., 2007). However, other studies have reported that exposure to VZ led to a reduction of gonadosomatic index (GSI) and retarded oocyte maturation in female fathead minnows, but only caused minimal effects in males (Martinovic et al., 2007).

### **1.3 WWTPs, MWWEs and Receiving Waterbodies in Saskatchewan**

#### **1.3.1 Surface Water Issues in Saskatchewan**

Southern Saskatchewan has a variety of surface water issues due to its uniqueness of being a prairie ecosystem as well as having experienced significant population growth in recent years. This rapid population growth has resulted in increasing amounts of discharges of effluents such as MWWEs into receiving water bodies. Furthermore, very little is known about the potential effects of municipal effluents such as stormwater or MWWEs in receiving water bodies in southern Saskatchewan. Additionally, there is particular pressure that sewage places on these waterbodies as many smaller communities do not have modern treatment technologies and need to rely on outdated lagoon systems.

There are unique factors that need to be considered when assessing contaminants in prairie surface water systems. Southern Saskatchewan is a semi-arid region, which results in seasonal drying up or low water flows of streams and low water levels of rivers that can result in low dilution of effluents in some seasons. For example, Wascana Creek in Regina, SK can have flows of up to



99% effluent during the dry seasons (Waiser et al., 2011a,b). Another unique factor of the Canadian prairie eco-region is the temperature regime of dry hot summers and extremely cold and long winters. This can significantly impact the efficiency of the biological treatment processes in WWTPs during the winter months, especially for lagoon-based treatment systems, and thus, might result in greater loading of contaminants and nutrients, including EDCs, into receiving water bodies. Thus, freshwater systems in the Canadian prairie region as well as similar regions around the world may be at elevated risk with regard to the exposure of emerging contaminants such as EDCs.

### **1.3.2 Wascana Creek, Regina, Saskatchewan, Canada**

Wascana Creek, Saskatchewan, Canada is an effluent-dominated system, which received treated sewage effluent from the city of Regina's (population ~ 230,000) outdated lagoon-based wastewater treatment facility until 2016. Located 3km west of Regina, the WWTP was a three-stage system consisting of bar screen and grit removal as primary treatment, a five-cell lagoon system (one month residence time) to remove solids and other pollutants through aeration and bacterial methods followed by seasonal ultraviolet disinfection (April – November) and alum treatment to remove phosphorus (Waiser et al., 2011). This WWTP discharges its effluent into Wascana Creek at an average rate of  $0.9\text{m}^3/\text{s}$ , and in winter, the treated sewage effluent makes up almost 100% of the creek flow until it connects with Qu'Appelle River 60km downstream (Waiser et al., 2011). The WWTP was built between 1956 and 1981, and was upgraded throughout its operation period, but it was built to serve a smaller Regina and to meet less comprehensive environmental standards. The plant in 2014 was considered an inadequate lagoon system that failed to meet new water quality regulations, raising significant concerns (City of Regina, 2014). Therefore, significant upgrades were planned, and the plant has since undergone upgrades

completed in December 2016 increasing the treatment capacity to 156ML/d of full treatment and 197ML/d for a short duration, versus the maximum 100ML/d that existed prior (<https://www.epcor.com/products-services/infrastructure/Pages/regina-wastewater-project.aspx>). In addition, a nutrient removal treatment process was added to remove both nitrogen and phosphorous. During the time of this study (2014-2015) the plant was still operating under its pre-upgrade conditions.

#### **1.4 Fathead Minnow as Test Species for EDCs**

Small fish species such as the fathead minnow (*Pimephales promelas*), Japanese medaka (*Oryzias latipes*), and zebrafish (*Danio rerio*) are commonly used as model organisms for regulatory screening and testing of endocrine disrupting chemicals worldwide (Ankley and Johnson, 2004). Of these species, the fathead minnow (FHM) represents a small fish model first used for studies in the 1950's, and it has since become the most widely used small fish model for regulatory ecotoxicology in North America due to their many attributes. It is a temperate freshwater fish distributed through much of North America, including Wascana Creek located in Southern Saskatchewan. Furthermore, they are ecologically important and rather faithful to their habitat, not migrating over long distances, making them a desirable sentinel species for *in situ* assessments. Fathead minnows are commonly used in a variety of studies to explore both exposure and effects of contaminants in both field and laboratory settings (Ankley, 2006). Additionally, they are able to tolerate a wide range of water quality conditions such as pH, hardness, alkalinity, turbidity and temperature making them easy to maintain in a laboratory setting (McCarraher and Thomas, 1968; Ankley, 2006). Finally, there is a vast knowledge of the fathead minnow's biology, behavioural patterns, reproduction, and life stages. While their basic biology has been studied exhaustively, only in the past decade research has begun on their basic reproductive physiology

and development, characterizing key parameters such as gonadal staging and sex steroids along the reproductive endocrine axis of these fish (Harries et al, 2000; Jensen et al, 2001). Specialized work regarding sexual development as well as identifying and characterizing the genes and proteins involved in the development and reproductive processes (Korte et al, 2000; Halm et al, 2001; Halm et al, 2004, Villeneuve et al, 2005) aids in determining and better understanding effects caused by exogenous chemicals such as EDCs. In parallel to their use in regulatory assessment, fathead minnows are used in a wide array of research applications. This species was used in the development of quantitative structure activity relationships (QSAR) models, and is commonly used for mixture toxicity as well as extrapolating effects of compounds across species and being able to extrapolate results from lab assays to the field setting (Ankley, 2006). Its past and current use has been extensive for both regulatory testing and research. In 2009, as part of the Endocrine Disruptor Screening Program (EDSP), the US-EPA developed standardized test guidelines for a fish short-term reproduction assay using the fathead minnow. Additionally, the OECD developed a similar 21-day fathead minnow reproductive assay for the testing of endocrine disrupting compounds. Furthermore, this species is used in tests ranging from acute lethality through partial and full life-cycle (Ankley, 2006). Its use in a variety of tests makes it valuable for regulatory programs aiming to assess potential risks of new chemicals such as pesticides, as well as complex mixtures like effluents.

## **1.5 Biological Endpoints of Endocrine Disruption in the Fathead Minnow**

Biological endpoints related to reproductive fitness and endocrine function are routinely used in the fish short-term reproduction assay of the US EDSP to assess the effects of endocrine disrupting compounds on fish, such as the fathead minnow. They are useful tools in EDC studies that may provide information on the mechanism of action of these compounds, physiological

changes and overall health and survival. Commonly assessed endpoints include steroid hormone homeostasis, organosomatic indices, growth, expression of target genes involved with reproduction, and histopathology, the latter of which provides insights into changes in maturation stages and possible structural alterations of the gonads such as intersex. It has been suggested that the use of multiple biomarkers and bioassays in both laboratory and field studies is ideal for determining impacts of exposure to potential EDCs (Vos et al., 2000; Ankley et al., 2006).

### **1.5.1 Hypothalamic-Pituitary-Gonadal-Liver Axis in Fish**

The pathway by which sex steroid and corticoid hormones are produced in all vertebrates and some invertebrates is termed steroidogenesis. Sex steroid hormones are critical components of the reproductive endocrine system as they control gonadal maturation, reproductive behaviour and the development and expression of primary and secondary sex characteristics. Steroidogenesis is a complex biosynthesis pathway involving a series of steps converting cholesterol into sex steroid hormones such as estradiol (E2), testosterone (T) and 11-ketotestosterone (11KT), the most active reproductive androgen specific to fish, and is controlled by the hypothalamus-pituitary-gonadal-liver (HPGL) axis (Arukwe, 2008; Hogan et al., 2010; Kime, 1993). Briefly, an external seasonal or local cue signals the brain, releasing gonadotropin releasing hormone (GnRH) from the hypothalamus, causing luteinizing hormone (LH) and follicle stimulating hormone (FSH) to be released from the pituitary where they act on the gonad to stimulate sex steroid hormone production (Kime, 1998). This pathway is tightly regulated by feedback loops that up- and down-regulate development, growth and reproductive processes, and which can be disrupted through the disruption of the expression or activity of enzymes and target genes involved in the HPGL axis (Ankley and Johnson, 2004). Specifically, target genes that mediate the synthesis of enzymes that produce gonadotropins released in the hypothalamus and pituitary include androgen receptor (AR),

estrogen receptors (ER $\alpha$  and ER $\beta$ ) and aromatase (CYP19 $\beta$ ). In the gonads, cholesterol transport and steroidogenesis involve luteinizing hormone receptor (LHR) and follicle-stimulating hormone receptor (FSHR) and enzymes such as StAR, 17 $\beta$ hsd and aromatase (CYP19 $\alpha$ ). And lastly in the liver, production of vitellogenin, an egg yolk protein precursor in fish, includes hormone receptors (AR, ER $\alpha$  and ER $\beta$ ) and key gene VTG (Ankley and Johnson 2004). VTG is regulated by ERs, making it an excellent biomarker of exposure to estrogenic chemicals and complex mixtures, such as municipal effluents.

Disruption of the actual process of steroidogenesis or steroidogenic tissues can alter the transcription of steroidogenic enzymes and their activity, resulting in alterations in the homeostasis of E2, T, 11KT or progesterone concentrations, which ultimately can lead to disruption of the reproductive endocrine system and the process of reproduction. Furthermore, these sex steroid hormones are also involved in processes not directly associated with the gonads such as immune function, brain development and behaviour (Arcand and Benson, 1997; Hecker et al., 2006). Therefore, it is not surprising that changes in the abundance of genes involved in steroidogenesis or circulating levels of these hormones may greatly impact sexual development, reproduction and growth.

Chemicals such as certain pesticides (e.g. dichlorodiphenyltrichloroethane [DDT] and methoxychlor), plasticizers (e.g. Bisphenol A and phthalates), certain dioxin-like compounds, polychlorinated biphenyls and polycyclic aromatic hydrocarbons (e.g. benzo[a]pyrene) as well as certain pesticides have been found to have the ability to alter the synthesis and metabolism of ovarian sex steroid hormones by altering the expression and catalytic activity of enzymes involved in steroidogenesis (Hecker et al. 2011; Craig et al., 2011). Exposure studies with MWWE showed that serum testosterone levels were significantly reduced in male fish (Folmar et al., 1996; Folmar

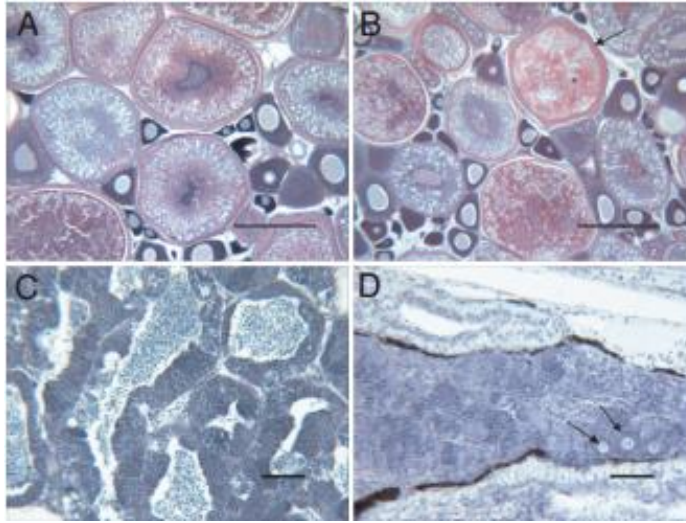
et al., 2000) and estrogen levels were significantly increased in both males and females (Folmar et al., 2000; Tilton, et al., 2002; Tetreault, G., 2012). Female fathead minnows exposed to prochloraz showed decreased gonadal E2 production and decreased plasma concentrations of VTG (Ankley et al., 2005, 2009). Both the inhibition of VTG, used as a relevant indicator for impaired maturation and reduced fecundity and the induction of VTG, particularly in males, considered to be a highly sensitive biomarker of estrogenic exposure in fish, have been used extensively worldwide, in both the laboratory and field settings (Sole et al., 2002; Routledge et al., 1998; Hashimoto et al., 2000; Vajda et al., 2008; Folmar et al., 1996; Palace et al., 2002; Kirby et al., 2004).

Due to the complexity of the HPGL, there are numerous potential targets for disruption. Therefore, analyzing the abundance of multiple genes involved in steroidogenesis in the HPGL axis as well as circulating levels of sex steroid hormones would be effective in evaluating the effects of EDCs on fish reproductive health (Ankley et al., 2012; Dang et al., 2016).

### **1.5.2 Histopathology of Gonads**

Histopathology is beneficial to the understanding and assessment of the potential pathological effects caused by EDCs in fish, as well as other organisms, making it a common biological endpoint in assessing EDCs. Exposure to endocrine disrupting compounds can have different effects on tissue structure and development depending on whether the fish is female or male. In the male, the occurrence of female germ cells (testicular oocytes; Figure 2D) or female tissue (mixed sex or ovo-testis) are the major evidence of fish feminization as a result of exposure to estrogenic chemicals or compounds that can stimulate endogenous estrogen production (Vos et al., 2000). The mixed sex condition in male fish is defined as testes containing a significant proportion of female ovarian tissue (Hecker et al. 2006). Since gonadal physiology and development for both the ovary and testes have been well established for fathead minnows,

changes from the normal gonadal histology in reproductively active female and male fathead minnows are easily defined.



**Figure 1.1.** Sample gonadal micrographs from fathead minnow. (A) Reference ovary with small, dark, primary-stage oocytes situated between cortical alveolar-stage oocytes and large vitellogenic oocytes. (B) EE2 exposed ovary displaying atretic follicle (arrow). (C) Reference testis. (D) EE2 exposed testes demonstrating testicular oocytes: arrows indicate primary-stage oocytes among the remnants of testicular tissue (from Kidd et al., 2007).



In males, the presence of oocytes in testes from exposure to estrogenic compounds has been well documented (Jobling et al., 1998; Vos et al., 2000; Wester et al., 1985). For example, up to 100% of male roach (*Rutilus rutilus*) exposed to estrogenic effluents from sewage treatment plants showed some form of mixed sex (ovo-testis; Jobling et al., 1998). Kidd et al. (2007), found that following the addition of the synthetic estrogen EE2 to an experimental lake at a concentration found in untreated and treated municipal wastewaters (4.8-6.1 ng/L), 4 of 9 male fathead minnows had testicular oocytes with the presence of primary-stage oocytes.

Exposure to MWWs has also been shown to affect normal testicular maturation resulting in altered reproductive capacities of males. A recent study with male fathead minnows exposed to treated sewage effluent found alterations in spermatogenesis, and in some cases total inhibition, with gonadal recrudescence, while the controls were at maturing stages (Diniz et al., 2005). Additionally, male white suckers (*Catostomus commersoni*) sampled downstream of a WWTP in Boulder, CO, USA had significantly reduced sperm abundance compared to fish collected at upstream sites (Vajda et al., 2008). Furthermore, male fathead minnows sampled downstream of the City of Regina's WWTP had relatively more spermatocytes and significantly fewer spermatogonia compared to reference males (Tetreault et al., 2012). Tetreault et al. (2012) observed that during the spawning season male fathead minnows had a significantly greater proportion of the later stage spermatids and spermatozoa, and significantly less spermatogonia than male fish from reference sites, suggesting a delay in the spawning activity of male fish downstream of the WWTP. Wild male FHMs exposed to environmentally relevant concentrations of EE2 (4.8-6.1 ng/L) exhibited delayed spermatogenesis, widespread fibrosis and malformations of the tubules compared to reference males (Kidd et al., 2007).

Similarly, in females, exposure to EDCs associated with MWWs can alter the developmental stage in the ovaries (Figure 2B). For example, in ovaries collected from fathead minnows downstream of a WWTP in Saskatchewan, Canada, the oocyte development appeared to be delayed compared to reference females (Tetreault et al., 2012). Additionally, following exposure to 50 and 100% effluent from an urban tertiary treatment plant in Portugal, follicular atresia was detected in female mirror carp (Diniz et al., 2005).

### **1.5.3 Secondary Sexual Characteristics**

During the breeding season, male fathead minnows develop a soft, mucus-secreting dorsal pad (fat pad), nuptial tubercles and dark banding becomes present (Ankley, 2006; Harries et al., 2000). These secondary sexual characteristics develop when the gonadotropic hormones, follicle stimulating hormone (FSH) and luteinizing hormone (LH), stimulate the gonads to produce androgenic hormones (Moyle and Cech, Jr., 1988). These developments are androgen dependent and have been shown to be highly responsive to a range of endocrine disrupting compounds (Hutchinson, 2006). For example, secondary sexual characteristics can be induced in males and females with exposure to androgens (Harries et al., 2000).

Nuptial tubercles, used in territory defense, are normally present on the head of reproductively active males, but with exposure to environmental androgens it has been shown to cause the development of facial tubercles on females and increase the expression in males (Ankley, 2001; Smith, R. 1974). Other studies have shown that exposure to estrogenic compounds can reduce the number and/or size of the tubercles in mature males (Miles-Richardson et al., 1999; Harries et al., 2000). Furthermore, atrophy of the breeding tubercles has been shown in FHM exposed to 17 $\beta$ -estradiol (Miles-Richardson et al., 1998), and is considered a useful indicator of environmental estrogen exposure (Miles-Richardson et al., 1999). The appearance and presence

of tubercles are useful indicators of endogenous androgen levels and are advantageous as they are visible externally and can be counted without injury to the fish (Smith, 1974).

The dorsal pad of FHM, used to clean the spawning surface and to rub over the developing embryos (Smith, 1978), is composed primarily of mucous cells in a mucinous matrix that is bound by a thickened epidermis (Smith, 1978). Atrophy of the fatpad has been shown after exposure to estrogenic compounds such as E2 (Miles-Richardson et al., 1999).

#### **1.5.4 Organosomatic Indices – GSI, HSI, K**

While molecular and histological biomarkers, described above, are useful as sensitive indicators of exposure to endocrine-disrupting chemicals, changes in organosomatic indices (OSIs) can also provide important information regarding exposure to toxicants. More importantly, they are considered vital biological information due to their ability to predict health issues of an individual that can be used to predict potential population level effects. Condition indices are a way to measure overall health by comparing the organism's weight and length relationship, or that of specific tissues with the typical weight of other fish of the same kind and length. Condition factor (K), a general quantitative health parameter, is calculated as  $\text{weight}/\text{length}^3$ . OSIs include gonadosomatic index (GSI) and hepatosomatic index (HSI). OSIs are calculated as (Eq. 1);

$$\text{OSI} = (\text{organ weight}/\text{total body weight}) * 100 \text{ (1)}$$

Condition factor (K) is a quantitative parameter of the overall health of the fish and reflects its feeding condition measured by comparing the fish's overall body weight to its length. Lower condition factors have been observed in males with exposure to sewage effluents (Lavado et al., 2003). Gonadosomatic index (GSI) is a calculation of the gonad mass as a proportion of the total body mass. It is useful for measuring the sexual maturity of fishes with regards to their ovary or

testes development. Significant changes in GSI with exposure to certain compounds can have deleterious effects on reproduction. GSI is a good indicator of the effects from exposure to endocrine disrupting compounds, and fish exposed to 100% waste water treatment effluents, which often contain EDCs, have been shown to have significantly decreased GSIs in both male and female fish (Diniz et al., 2005). While a low GSI is not necessarily an indicator of exposure to EDCs as reports have shown decreases with exposure to non-estrogenic contaminants such as dioxin contaminated effluents (Sakamoto et al., 2003), it is most often linked to a pathological alteration in the gonads (Kinnberg et al., 2000).

Hepatosomatic index (HSI) is another measurement of general health and is the liver mass represented as a proportion of the total body mass. It provides an indication on the status of energy reserves in an animal and because contaminants are actively metabolized in the liver, a high HSI value could be a sign of increased metabolic activity due to contaminant exposure. Furthermore, because vitellogenin is produced in the liver, an increase in vitellogenin production could increase relative liver weight (Hemming et al., 2001). In a recent study with mirror carp (*Cyprinus carpio*) exposed to treated municipal sewage effluent, all exposed fish had elevated HSIs compared to control animals (Diniz et al., 2005). Increases in HSI associated with liver hypertrophy indicate that there are possible estrogenic effects from effluents sewage treatment plants due to marked increases in hepatic vitellogenin production (Hemming et al., 2001). In summary, GSI and HSI can be useful indicators of the effects from exposure to endocrine disrupting compounds.

## **1.6 Objectives and Hypotheses**

Effluents from WWTPs typically contain complex mixtures of chemicals including EDCs. Because conventional wastewater treatment technologies are often incomplete or inefficient at removing these contaminants, effluents containing EDCs are released into receiving water bodies.

MWWEs are becoming a particular concern in arid or semi-arid regions such as southern Saskatchewan as receiving water bodies in the southern prairies are often small and can consist of up to 100% effluent during dry seasons. Compounds present in these effluents can act through agonistic or antagonistic binding to nuclear steroid hormone receptors, through altering sex steroid homeostasis or other mechanisms of toxicity. While the former can result in up- or down regulation of associated genes, respectively, as well as pathological alterations (e.g. mixed sex condition or inhibition of maturation), disruption of steroidogenesis can lead to altered hormone levels, abnormal gonad morphology, delayed sexual maturity or reproductive failure in fish (Palace et al., 2002; Folmar et al., 1996; Jobling et al., 1998; Miles-Richardson et al., 1999). Despite all of these concerns, very little is known about the potential impacts of EDCs associated with MWWEs to local aquatic ecosystems in the Canadian prairies. Therefore, the overall purpose of this study was to characterize the potential endocrine effects of MWWEs released into Wascana Creek, Saskatchewan to a native fish species, namely the FHM. Results from this work were compared with targeted chemical analysis and results from *in vitro* bioassays and *in vivo* laboratory studies (data obtained during two parallel studies) as part of a larger overall project known as Aquatic Impact Assessment of Municipal Effluents (AIME). This multi-tiered study aimed to characterize the presence of chemicals with endocrine activities in MWWEs in Canada and to assess any potential impacts of these effluents on aquatic species in the receiving environments, ultimately developing a “toolbox” to efficiently, economically and reliably prioritize MWWEs of concern. Successful completion of this specific project will provide valuable information on the current ecological risks associated with MWWEs in the Canadian prairie region, and will inform future needs for developing more efficient and effective approaches for eliminating EDCs from MWWEs.

**Objectives:**

The **overall objective** of this study was to evaluate the potential impacts of MWWEs on reproductive health and endocrine homeostasis of wild fathead minnow (*Pimephales promelas*) populations downstream of the City of Regina's WWTP in Wascana Creek, Saskatchewan, Canada. Specific **objectives** were:

- 1) Compare maturation status, reproductive health and endocrine status between populations of FHMs collected up- and down-stream of the city of Regina.

H<sub>0</sub>1: There is no statistically significant difference in maturation status, reproductive health and/or endocrine status between populations of FHM collected up- and down-stream of Regina.

- 2) Compare maturation status, reproductive health and endocrine status of wild FHM between two sampling years (2014 and 2015).

H<sub>0</sub>2: There is no statistically significant difference in maturation status, reproductive health and/or endocrine status between sampling years (2014 and 2015).

- 3) To determine if effects observed in FHM populations downstream, if any, are correlated to exposure to certain chemicals present in municipal wastewater effluents released from the City of Regina's wastewater treatment plant during time of sampling.

H<sub>0</sub>3: There are no statistically *significant* correlations between biomarker responses or health effects in FHM populations downstream of the MWWEs and exposure to select chemicals of concern.

**Chapter 2 : HEALTH STATUS OF FATHEAD MINNOW  
(*PIMEPHALES PROMELAS*) POPULATIONS IN A MUNICIPAL  
WASTE WATER EFFLUENT-DOMINATED STREAM IN THE  
CANADIAN PRAIRIES, WASCANA CREEK, SK.**

## PREFACE

There is limited information regarding the potential effects of municipal waste-water effluents, and the endocrine disrupting compounds within them, on aquatic species in the Canadian prairie region. Therefore, the aim of Chapter 1 was to investigate the status of health of resident fathead minnow populations in an effluent dominated system, Wascana Creek, SK, Canada. This chapter was organized as a manuscript for publication in a peer-reviewed scientific journal.

### Author Contributions:

Sara Hanson (University of Saskatchewan) designed and managed the experiment, generated and analyzed all data, and drafted the manuscript.

Tabata Bagatim (University of Saskatchewan) conducted a parallel study on the endocrine potentials municipal effluents from two prairie WWTPs, providing information on *in vitro* studies to correlate with *in vivo* results.

Kean Steeves (University of Saskatchewan) conducted a parallel study on the endocrine system of laboratory fish exposed to municipal effluent from two prairie WWTPs, providing information on laboratory *in vivo* studies to correlate with field based *in vivo* results.

Steve Wiseman (University of Lethbridge) provided guidance throughout experiments and offered comments and edits to the manuscript.

Natacha Hogan (University of Saskatchewan) provided guidance throughout *in vitro* experiments and offered comments and edits to the manuscript.



Alice Hontela (University of Lethbridge) provided guidance throughout *in vitro* experiments, offered comments and edits to the manuscript.

Paul Jones (University of Saskatchewan) provided guidance throughout chemical analysis experiments, as well as offered comments and edits to the manuscript.

John Giesy (University of Saskatchewan) provided the molecular laboratory to conduct the mechanistic portion of study and offered comments and edits to the manuscript.

Markus Hecker (University of Saskatchewan) provided guidance and inspiration for the conception and design of the experiment, offered comments and edits to the manuscript, and provided research funding.

## 2.1 Abstract

Waterbodies in the southern Canadian Prairies may be at an elevated risk to the exposure with contaminants released within municipal waste-water effluents (MWWEs), including endocrine disrupting compounds (EDCs), due to the uniqueness of prairie surface water systems. For example, during low flow periods, Wascana Creek, SK can contain up to 100% treated effluent downstream of the City of Regina's outdated lagoon based treatment facility. The aim of this study was to characterize the potential endocrine disrupting effects of municipal waste-water effluents on wild fathead minnow (*Pimephales promelas*; FHM) populations in an effluent dominated stream, Wascana Creek, SK. Field studies were conducted on spawning FHMs (2014 and 2015) to assess responses in terms of overall health (condition factor, ovosomatic indices) and reproduction (secondary sexual characteristics, gonad histopathology). FHMs downstream of the effluent fallout had lower gonadosomatic indices and significantly greater hepatosomatic indices compared to upstream populations. In both male and female FHMs significantly greater occurrence and severity of gonadal degradation and delayed maturation were observed in downstream fish compared to upstream fish; however, no indications of exposure to estrogenic compounds such as occurrence of testicular oocytes were observed. Furthermore, exposed males displayed lower scores of secondary sexual characteristics. Chemical analysis indicated that 10 PPCPs were present downstream, some at sufficiently great concentrations that may present a risk to aquatic organisms. With continuous exposure to a diverse number of stressors including high nutrient and ammonia levels and presence of a variety of PPCPs and other contaminants, Wascana Creek should be considered as an ecosystem at risk with regard to effects from MWWEs on resident fish populations. While this study was regional in nature, due to similarities, inferences can likely be made among different semi-arid regions around the world in regards to the ecological impacts of MWWEs.

## **2.2 Introduction**

In recent decades, concern has been raised surrounding the occurrence of emerging contaminants (ECs) in our water resources and the effects they may have on resident organisms. ECs are a diverse class of compounds including, but not limited to, pesticides, soaps and detergents, plasticizers, nanoparticles, brominated flame retardants, personal care products and pharmaceuticals (PPCPs) as well as hormones and chemicals released from livestock operations (Purdom et al., 1994; Folmar et al., 1996; Hoffman and Oris., 2006). These compounds are able to enter the aquatic environment through many different routes such as agricultural runoff, release of industrial effluents, and municipal waste water effluents (MWWWE), with the latter representing one of the most prominent sources of these compounds (CCME, 2006). MWWWEs contain complex mixtures of not only organic waste, nutrients, and pathogens, but also a suite of ECs such as steroidal estrogens, PPCPs and household and industrial chemicals that are not or only partially removed during the treatment process (Desbrow et al., 1998; Mills and Chichester, 2005; Baronti et al., 2000). Many ECs present in MWWWEs have been identified as endocrine disrupting compounds (EDCs) that have been shown to affect growth, development and reproduction of aquatic organisms, particularly fish, through disruption of endocrine homeostasis, sometimes at very low concentrations (Campbell et al. 2006; Hecker et al., 2006; Goksøyr 2006).

The effects of MWWWEs on wild fish have been well studied throughout both Europe and North America with estrogenic impacts in male fish downstream of WWTPs resulting in the presence of female germ cells in testicular tissue being one of the most frequently reported phenomena (Purdom et al., 1994; Sumpter and Jobling, 1995; Jobling et al., 1998; Kidd et al., 2007). Together with other reproductive impairments such as delayed gonadal maturation and development as well as impacts on sex steroid hormone levels, this intersex condition has been

widely observed around the world in wild fish exposed to MWWs (Jobling et al., 1998; Hecker et al., 2002). In a 7-year whole-lake study, Kidd et al. (2007) assessed the effects of the potent synthetic estrogen 17 $\alpha$ -ethinylestradiol (EE2) on fathead minnow (*Pimephales promelas*) populations. They showed that low chronic exposure (5 ng/L), similar to levels occurring in aquatic systems with continuous inputs of MWWs, led to feminization of males, histological alterations evidenced by intersex in males and altered oogenesis in females and population changes with a near extinction of the species from the experimental lake (Kidd et al., 2007). In addition to estrogenic effects, exposure to other EDCs, such as those with anti-estrogenic, and (anti)-androgenic properties need to be considered for their potential to contribute to disruption of the reproductive system. Varying responses have been observed for several compounds often found in MWWs. For example, decreases in plasma vitellogenin as well as fecundity and fertility were observed in female Japanese medaka (*Oryzias latipes*) exposed to the anti-estrogen, tamoxifen (Sun et al., 2007). Furthermore, reduced testosterone levels occurred in male fathead minnows exposed to the herbicide linuron and the plastizer DEHP (anti-androgens; Crago and Klaper, 2012), and reduced fecundity and formation of secondary sexual characteristics were reported in female fathead minnows exposed to 17- $\beta$ -trenbolone (androgen; Ankley et al., 2003). In addition to the effects on gonadal development and maturation, sex steroid homeostasis and expression of secondary sexual characteristics, exposure of fish to EDCs have also been shown to alter organosomatic indices. Lower condition factors (Lavado et al., 2003), decreased gonadosomatic indices (GSI), and elevated hepatosomatic indices (HSI; Diniz et al., 2005) have been observed in male and female fish after exposure to MWWs.

Although concentrations of ECs are generally low in treated sewage effluents, environmental risks are still present due to the pseudo-persistence of these chemicals (continuous

influx). This is of particular concern in semi-arid environments, like the Canadian prairies, where receiving water bodies are often characterized by low flow and minimal dilution of effluents (Cwiertny et al., 2014; Dotan et al., 2016; Waiser et al., 2011). In addition to the minimal dilution of effluents, prairie ecosystems, such as occur in Southern Saskatchewan, are also characterized by an extreme temperature regime, that changes drastically throughout the year (+45°C to -50°C), but also between day and night. This can potentially impact the efficiency of biological treatment processes in wastewater treatment plants (WWTPs) during the winter and spring months, resulting in greater loading of contaminants and nutrients including EDCs into receiving surface water systems. This is of particular concern with regard to outdated lagoon systems that are still common practice in this area. In addition, a rapid population growth is currently occurring in the prairie provinces of Canada, resulting in increasing amounts of discharges of effluents that many WWTPs are not equipped to handle.

However, even though potential risks have been identified, little information is known regarding the potential effects of MWWEs to surface water systems in the Canadian prairies (Waiser et al., 2011). In fact, a number of steppe areas with similar cold semi-arid climates exist globally for which no or only limited information regarding the impact of MWWEs is available to local aquatic ecosystems, including the central United States, Asia, Eastern Europe, and sections of South America. Due to their similarities, inferences can likely be made among different semi-arid regions around the world in regards to the ecological impacts of MWWEs.

Wascana Creek, Saskatchewan, Canada, was chosen as a representative system for small semi-arid and cold prairie environments, as it is an effluent dominated prairie stream that receives MWWEs from the City of Regina's outdated lagoon-based treatment facility. The treatment plant discharges effluent into Wascana Creek at a rate of 0.90m<sup>3</sup>/s, and in winter, the treated sewage

effluent can make up to 99% of the creek flow until it connects with the Qu'Appelle River 60km downstream (Waiser et al., 2011). Previous studies in Wascana Creek, SK have shown the presence of 25 PPCP's and their metabolites in the ng/L to µg/L range downstream of the effluent fallout as well as excessive nutrients being released within the effluents, causing it to be a nitrogen hypersaturated system (total dissolved N > 3 mg/L; Waiser et al., 2011). Additional organismal studies observed delayed spawning, and altered gonadal development in Fathead Minnows downstream of the City of Regina's effluent fallout (Tetreault et al., 2011).

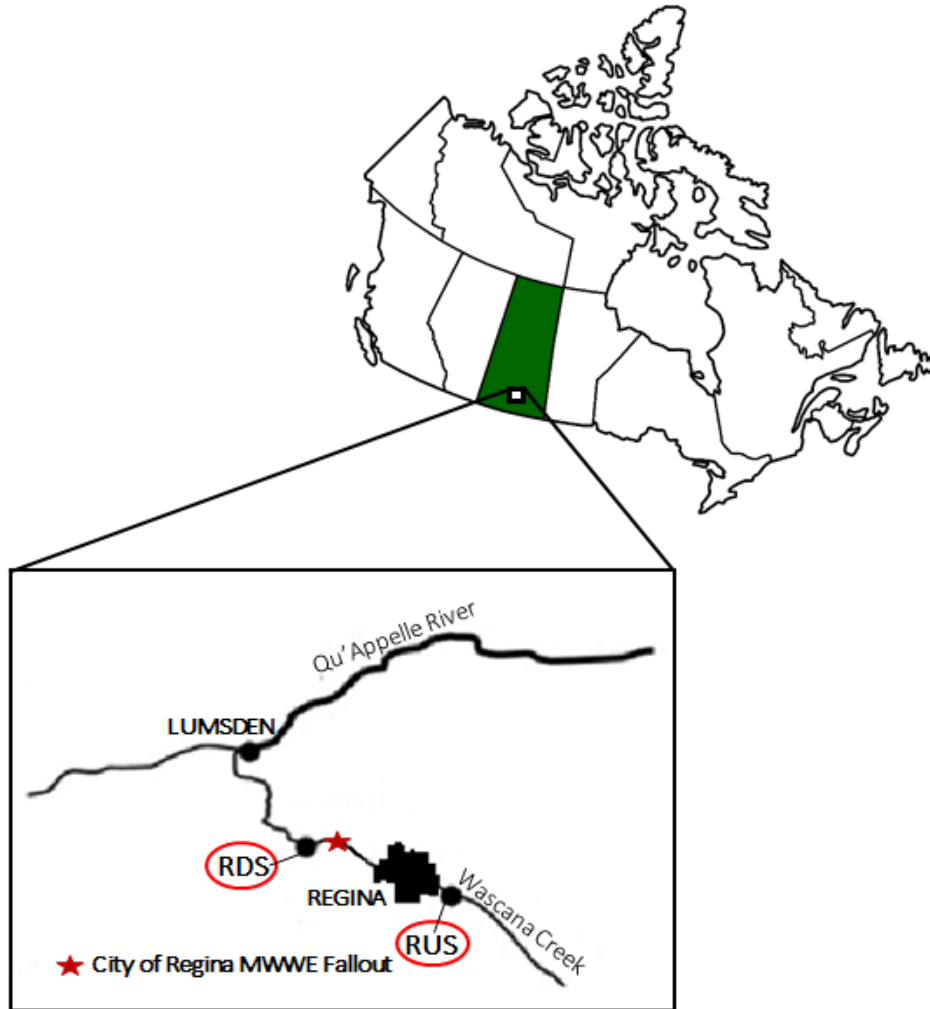
The overall aim of the present study was to investigate the overall health and reproductive status of wild fathead minnow populations in Wascana Creek, SK, Canada. Specifically, the present study discusses 1) overall health of fathead minnow populations in Wascana Creek, SK up- and downstream of the City of Regina's WWTP outfall; 2) observations and alterations of gonad histopathology in both males and females; 3) stable isotope analysis to determine if movement between the two populations is occurring; 4) chemical analysis of in stream samples to determine concentrations of EC's; and 5) whether any observed effects could be attributed to the EC's present.

## **2.3 Materials and Methods**

### **2.3.1 Site Location and Field Collections**

Fathead minnows were collected in 2014 and 2015 during spawning season (late July) from two locations in Wascana Creek, SK, Canada; a reference site upstream of the city (RUS; 50.40, -104.49), and directly downstream of the effluent fallout (RDS; 50.48, -104.75)(Figure 2.1). Seine nets were pulled from shore to shore and approximately 30 fathead minnows from each site were collected, removed and transported to an on-site mobile laboratory in aerated pails. During each collection event at both sites, surface water quality was measured using a YSI

Professional Plus meter with YSI Quatro ISE-ISEDO-COND-T probes (YSI, Yellow Springs, OH, USA) for temperature, dissolved oxygen, and pH.



**Figure 2.1.** Site locations for sampling of Fathead Minnows in Wascana Creek. RUS (Regina Upstream Site); RDS (Regina Downstream Site); MWW (Municipal wastewater effluent).



### 2.3.2 Tissue Sampling and Morphometrics

Immediately following sampling, fish were anesthetized in Aquacalm (5-10 mg/L; Syndel Laboratories, Nanaimo, BC, Canada). Mass (to the nearest 0.01 g) and fork length (to the nearest 0.1 cm) were determined and condition factor (Equation 1) was calculated. Fish were euthanized by spinal severance. Using the carcass, secondary sexual characteristics were scored based on the U.S. EPA Fish short-term reproduction assay test protocol – Fathead Minnow, and pictures were taken (USEPA 2009). Livers and gonads were weighed and hepatosomatic index (HSI, Equation 2) and gonadosomatic index (GSI, Equation 3), respectively, were calculated. Half of each gonad tissue was fixed in 10% buffered formalin for histopathological analysis.

$$\text{Condition Factor} = (\text{body weight}/\text{length}^3)*100 \quad (1)$$

$$\text{HSI} = (\text{liver weight}/\text{total body weight})*100 \quad (2)$$

$$\text{GSI} = (\text{gonad weight}/\text{total body weight})*100 \quad (3)$$

### 2.3.3 Histological Analysis

Gonads were placed in 10% buffered formalin and fixed for 24 hours. After proper fixation, the gonads were rinsed with, transferred to and stored in 70% ethanol at room temperature until further processing. The tissues were processed following standard histological methods (Appendix A) using an automatic tissue processor (Leica Microsystems Inc., Concord, ON, Canada) and embedded in paraffin wax. Processing was conducted by Prairie Diagnostic Services, University of Saskatchewan. Paraffin blocks were serially sectioned at 4 and 6µm for testes and ovaries, respectively, on a Microm HM model 310 microtome (GMI, Ramsey, MN, USA). Tissue sections were placed onto a 37°C water bath and then picked up on Superfrost Plus slides

(ThermoScientific, Pittsburgh, PA, USA). Slides were dried overnight on a slide warmer and were then stained with hematoxylin-2 and eosin-y (H & E) following standard histological methods (Appendix A) as outlined in the Fish Short-Term Reproduction Assay (US EPA 2011). Following staining, the sections were covered with cover slips and Microkitt xylene-based mounting medium (Serum International, Inc, Laval, QC, Canada) and dried for at least 24 hours.

Seven males and ten females from both the upstream and the downstream site were selected. From each of these fish, three slides with five sections each were analyzed on a Zeiss Axiostar Plus light microscope and photographs were taken using an AxioCam MRC 5 MP camera (Carl Zeiss Canada Ltd., Toronto, ON, Canada) and recorded using Axiovision 4.8 Imaging software (Carl Zeiss Canada Ltd., Toronto, ON, Canada). Primary criteria were scored for both sexes, using either a severity or staging index (see Appendix, Fig. C2.S1) in accordance with the Diagnosis of Endocrine-related Histopathology of Fish Gonads (US EPA 2009). Severity grading's (0 = non-remarkable, 1 = minimal, 2 = mild, 3 = moderate and 4 = severe) were assessed for each specimen and used for all primary criteria (see Appendix B). Staging criteria (juvenile, stage 0 = undeveloped, stage 1 = early development, stage 2 = mid-development, stage 3 = late development, stage 4 = late development/hydrated, stage 5 = post-ovulatory) were used to compare the relative maturational stage of the ovaries (see Appendix B).

Unfortunately, due to the low number of males collected in 2015 at RDS, there were insufficient tissues available for histological analysis of males during that year, so only 2014 data and 2015 RUS are discussed here.

### **2.3.4 Stable Isotope Analysis**

Spleen tissue was sampled from fathead minnows from both sites in both years and preserved in liquid nitrogen, transported to the Toxicology Centre, University of Saskatchewan (Saskatoon, SK, Canada) and then stored at -80°C until analysis. Spleen samples were analysed for stable isotopes of carbon ( $^{13}\text{C}/^{12}\text{C}$ ) and nitrogen ( $^{15}\text{N}/^{14}\text{N}$ ) by isotope ratio mass spectrometry and expressed as  $\delta$  values (eg.,  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$ ). EM Protein 1 and atmospheric nitrogen ratios (heavy to light) were used to derive standards for  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$ , respectively. Samples were dried, weighed, homogenized using a mortar and pestle and analyzed using a Costech ECS4010 elemental analyzer coupled to a Thermo Scientific Delta V mass spectrometer (Painter et al., 2015). Analysis was conducted by Miles Stocki Stable Isotope Facilities at the University of Saskatchewan.

### **2.3.5 Chemical Analysis**

Water samples were collected from each site in 2014 in two one litre amber bottles (total volume 2L) with two to three drops of chloroform added for preservation purposes and transferred to the Toxicology Centre on ice and then stored in the dark at 4°C until analysis. Samples were filtered and transferred to a new clean bottle. Solid phase extraction was conducted using two litres of sample and air dried following the addition of 5mL of MiliQ water and 0.1% acetic acid. Cartridges were stored at -20°C. Cartridges were extracted using 5mL of 1:1 Hexane:DCM, dried using nitrogen and glass tubes stored at -20°C. Samples were reconstituted by adding 400 $\mu\text{L}$  of isooctane for each 2L sample. Following reconstitution, extracts were transferred into a clear insert and placed into an amber vial and stored at -20°C until analysis.

Extracts were analyzed in house using a Q Exactive™ mass spectrometer (Thermo Fisher Scientific, Toronto, ON) interfaced to a Dionex™ UltiMate™ 3000 ultra-high-performance liquid chromatography (UHPLC) system (Thermo Fisher Scientific, Toronto, ON) with modifications (Hui et al., 2005). Separation of chemicals was achieved with a Betasil C18 column (5 µm; 2.1 mm × 100 mm; Thermo Fisher Scientific, Toronto, ON) with an injection volume of 5 µL. Ultrapure water (A) and methanol (B) were used as mobile phases. Initially 10% B was increased to 50% in 5 min, then increased to 100% at 20 min and held static for 6 min, followed by a decrease to initial conditions of 10% B and held for 3 min to allow for column re-equilibration. The flow rate was 0.20 mL/min. The column and sample chamber temperatures were maintained at 40 °C and 10 °C, respectively. Data was acquired using full scan mode and selected ion monitoring (SIM). Briefly, MS scans (100 - 1000  $m/z$ ) were recorded at resolution  $R = 70000$  (at  $m/z$  200) with a maximum of  $3 \times 10^6$  ions collected within 200 ms, based on the predictive automated gain control. SIM scans ( $m/z = 227.1072, 271.1698, 269.1542, 295.1698$ ) were recorded at a resolution  $R = 35000$  (at  $m/z$  200) with a maximum of  $5 \times 10^4$  ions collected within 80 ms, based on the predictive automated gain control, with the precursor isolation width set at 2.0  $m/z$ . General mass spectrometry settings applied for negative ion mode were as follows: spray voltage, 2.7 kV; capillary temperature, 375 °C; sheath gas, 46 L/h; auxiliary gas, 11 L/h; probe heater temperature, 375 °C. Similarly, settings applied for positive ion mode were: spray voltage, 3.0 kV; capillary temperature, 400 °C; sheath gas, 46 L/h; auxiliary gas, 15 L/h; probe heater temperature, 350 °C.

### **2.3.6 Statistical Analysis**

Statistical evaluation of the data was conducted using SPSS Version 20 (IBM Corp., Armonk, NY, USA). Male and female fish were analyzed separately. Normality of the data was tested using Shapiro-Wilk test and homogeneity of variance was tested using Levene's test. Any data that did not meet the assumptions of a parametric test were logarithmically transformed and re-analyzed for use in parametric tests. Where no normalizing transformation could be found, the Kruskal-Wallis test followed by the Mann Whitney U post hoc test were applied. Normally distributed data was analyzed using analysis of variance (ANOVA) followed by Tukey's post hoc test to determine if there was significant variation among and within the sampled sites for all parameters investigated. All statistical tests were performed using an alpha value of 0.05.

## **2.4 Results**

Surface water quality parameters measured at both sites were comparable between 2014 and 2015, with slightly higher temperatures and lower DO and pH values at the downstream site. The highest recorded temperature was in 2014 at the downstream site (25.4°C) and the lowest in 2014 at the upstream site (23.8°C). Dissolved oxygen ranged between 11.1 and 12.1 mg/L at the upstream site, and 10.4 and 10.8 mg/L at the downstream site in 2014 and 2015, respectively. pH varied slightly between sites, ranging between 8.45 and 8.49 at the upstream and 8.23 and 8.31 at the downstream site. Unionized ammonia concentrations in the final effluents released into the creek averaged 0.241 and 0.102 mg/L for July 2014 and 2015 respectively, with a maximum concentration of 0.511 mg/L detected in 2014 (Table 2.1). Total phosphorus concentrations in the effluents released ranged from 0.46 to 1.99 and 0.47 to 1.08 mg/L in July 2014 and 2015

respectively (Table 2.1). Nitrogen concentrations on average were lower in July 2014 (27.13 mg/L) compared to 2015 (38.5 mg/L) final effluents (Table 2.1).

**Table 2.1.** Average, maximum and minimum concentrations of ammonia nitrogen (mg/L), unionized ammonia at 15±1°C (mg/L), total phosphorus (mg/L) and total nitrogen (mg/L) in final effluents at the City of Regina's WWTP in July for both 2014 and 2015.

		<b>Ammonia Nitrogen (mg/L)</b>	<b>Unionized Ammonia at 15±1°C (mg/L)</b>	<b>Total Phosphorus (mg/L)</b>	<b>Total Nitrogen (mg/L)</b>
<b>July 2014</b>	Average	16.6	0.241	0.87	27.1
	Max	24.1	0.511	1.99	33.0
	Min	12.0	0.101	0.46	23.8
<b>July 2015</b>	Average	17.9	0.102	0.74	38.5
	Max	13.6	0.179	1.08	47.1
	Min	24.9	0.066	0.47	34.2

### 2.4.1 Fish Health

In 2014, there were no differences with respect to length, weight and condition factor ( $p=0.167$ ) of female fathead minnows between sites. However, RDS males were shorter and lighter, with significantly greater condition factors ( $p<0.01$ ) when compared to upstream males (Table 2.2). RDS female fish had significantly larger livers ( $p<0.001$ ) and approximately 4x smaller gonads, with significantly lower GSIs ( $p<0.05$ ) when compared to RUS females. Males collected downstream of the effluent fallout displayed significantly larger livers ( $p<0.001$ ) and significantly lower GSIs ( $p<0.05$ ) when compared to RUS males (Table 2.2).

Females collected in 2015 at RDS were significantly longer and heavier ( $p<0.001$ ) and had lower condition ( $p<0.001$ ) compared to females collected at RUS. No difference between sites in respect to length, weight and condition factor was found for males ( $p=0.117$ ) (Table 2.2). Female and male fathead minnows collected at RDS had significantly larger livers compared to RUS fish ( $p<0.05$ ). Significantly smaller gonads were seen for females downstream, compared to organ sizes of the upstream fish ( $p<0.01$ ). While gonads of downstream males were over 2x smaller than those of upstream males, this difference was not statistically significant ( $p=0.125$ ) (Table 2.2).

In both 2014 and 2015, males downstream of the effluent fallout had significantly lower average nuptial tubercle scores compared to males collected at the upstream site (2014:  $p<0.05$ ; 2015:  $p<0.01$ ). The presence of nuptial tubercles on female fathead minnows was not observed at any site during either season.



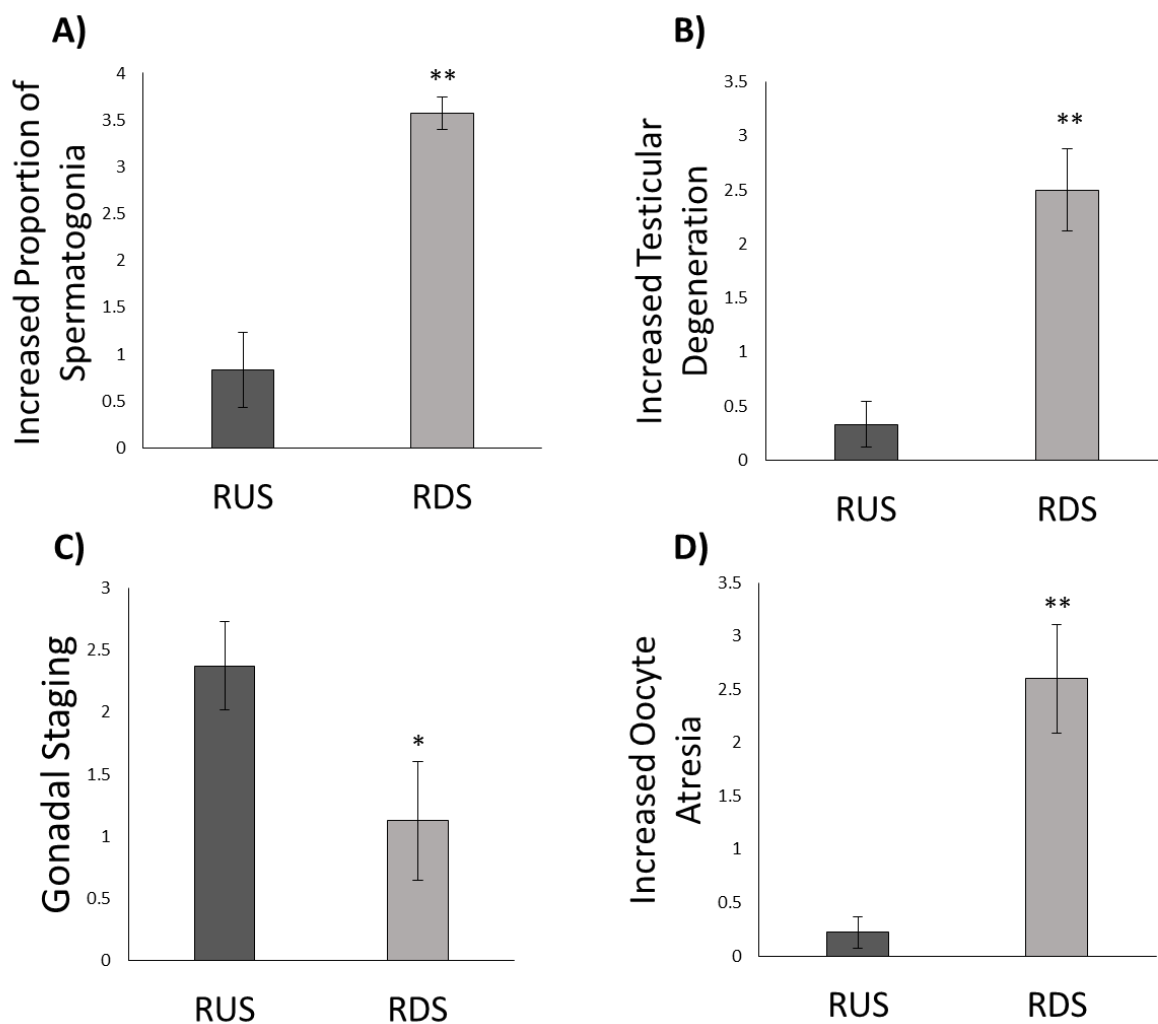
**Table 2.2.** Mean ( $\pm$  SE) (n) length, body weight, condition factor (k), hepatosomatic index (HSI), and gonadosomatic index (GSI) of Fathead Minnows collected in Wascana Creek, SK. Differences among sites ( $p < 0.05$ ) are denoted by different lowercase letters.

Year	Site	Sex	Length (cm)	Weight (g)	$k^a$	HSI <sup>b</sup>	GSI <sup>c</sup>
2014	RUS	Female	4.59 $\pm$ 0.09 (17) a	0.98 $\pm$ 0.06 (17) a	0.99 $\pm$ 0.04 (17) a	1.70 $\pm$ 0.84 (16) a	9.92 $\pm$ 3.18 (10) a
	RDS		4.92 $\pm$ 0.31 (10) a	1.33 $\pm$ 0.18 (10) a	1.10 $\pm$ 0.07 (10) a	3.74 $\pm$ 0.38 (9) b	2.21 $\pm$ 0.87 (9) b
	RUS	Male	5.37 $\pm$ 0.19 (10) a	1.63 $\pm$ 0.19 (10) a	1.02 $\pm$ 0.02 (10) a	1.57 $\pm$ 0.24 (9) a	0.96 $\pm$ 0.25 (8) a
	RDS		4.47 $\pm$ 0.16 (25) b	1.1 $\pm$ 0.11 (25) b	1.17 $\pm$ 0.03 (25) b	3.76 $\pm$ 0.24 (25) b	0.60 $\pm$ 0.04 (19) b
2015	RUS	Female	4.56 $\pm$ 0.10 (17) a	1.11 $\pm$ 0.06 (17) a	1.16 $\pm$ 0.04 (17) a	1.95 $\pm$ 0.18 (13) a	10.40 $\pm$ 1.03 (13) a
	RDS		5.42 $\pm$ 0.08 (26) b	1.5 $\pm$ 0.07 (26) b	0.93 $\pm$ 0.02 (26) b	2.69 $\pm$ 0.23 (26) b	3.99 $\pm$ 1.97 (7) b
	RUS	Male	4.97 $\pm$ 0.17 (6) a	1.64 $\pm$ 0.06 (6) a	1.38 $\pm$ 0.14 (6) a	1.30 $\pm$ 0.19 (6) a	2.21 $\pm$ 0.40 (5) a
	RDS		5.43 $\pm$ 0.37 (4) a	1.73 $\pm$ 0.36 (4) a	1.05 $\pm$ 0.10 (4) a	2.43 $\pm$ 0.37 (4) b	0.81 $\pm$ 0.66 (2) a

### 2.4.2 Histopathology

In 2014, males downstream of Regina displayed a significant increase in the proportion of the earlier cell type spermatogonia and lower mature spermatozoa when compared to males collected upstream with a minimal severity grade ( $0.83 \pm 0.37$ ) upstream and a moderate to severe severity grade ( $3.57 \pm 0.16$ ) downstream ( $p < 0.001$ , Figure 2.2, 2.3). There was no incidence of ovo-testis. Testicular degeneration, characterized primarily by apoptotic germ cell formation, was significantly increased in males at RDS compared to RUS ( $p < 0.001$ , Figure 2.2, 2.3). Exposed males also had a significant increase in the incidence of interstitial cell (Leydig) hypertrophy/hyperplasia compared to males collected upstream ( $p < 0.01$ ).

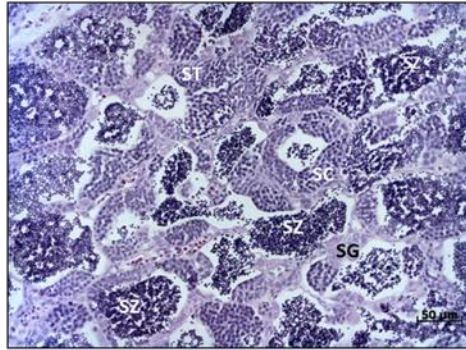
Histopathological analysis of ovaries in both 2014 and 2015 did not demonstrate any differences between the upstream and downstream sites for perfollicular cell hyperplasia/hypertrophy or decreased yolk formation. However, in both years, females at RDS did display significant increases in oocyte atresia compared to RUS (2014:  $p < 0.001$ , 2015:  $p < 0.01$ , Figure 2.2, 2.3). Also, RDS females were at a significantly lower gonadal maturation stage (stage 1) compared to RUS (stage 2.5) specimens in both 2014 ( $p < 0.05$ ) and 2015 ( $p < 0.01$ , Figure 2.2).



**Figure 2.2.** Average severity grading's ( $\pm$ SEM) for associated histopathological primary criteria of males (A and B), and females (D) at sampling locations upstream (RUS) and downstream (RDS) of the City of Regina in 2014. Average maturity grade of females (C). \* =  $p < 0.05$  and \*\* =  $p < 0.001$ .

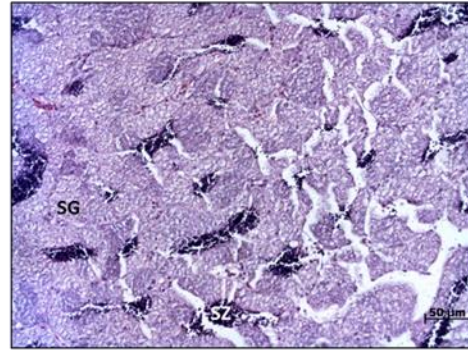
Upstream (RUS)

1a)

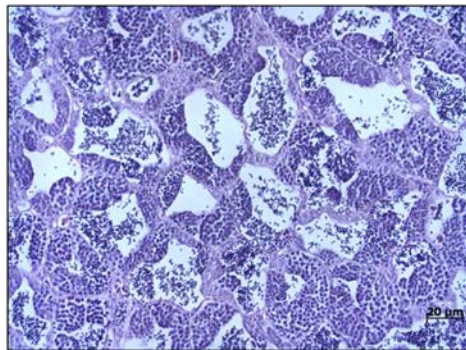


Downstream (RDS)

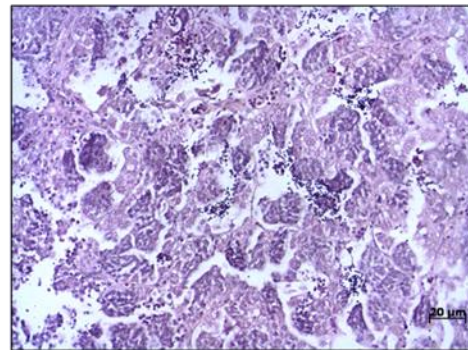
1b)



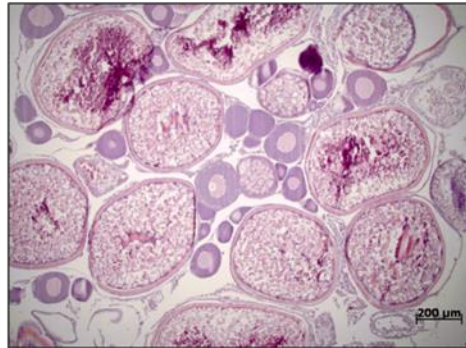
2a)



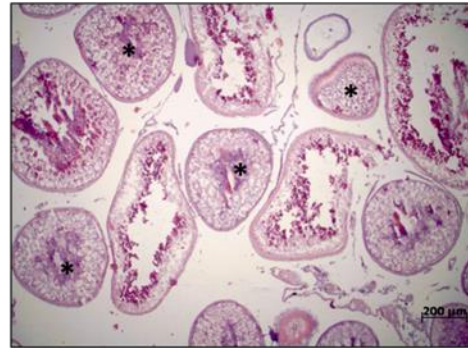
2b)



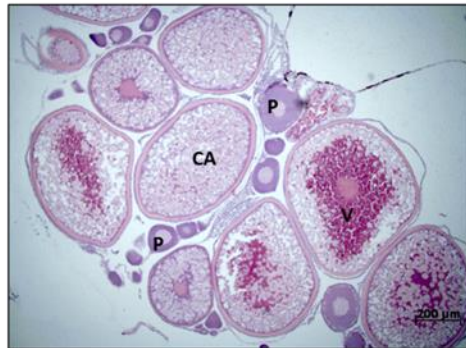
3a)



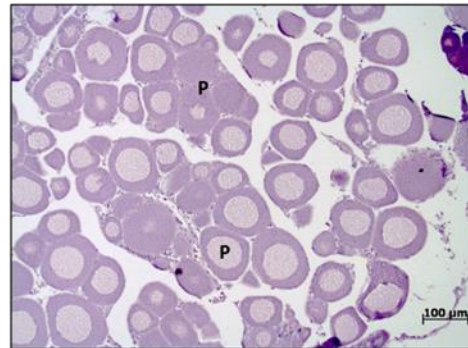
3b)



4a)



4b)

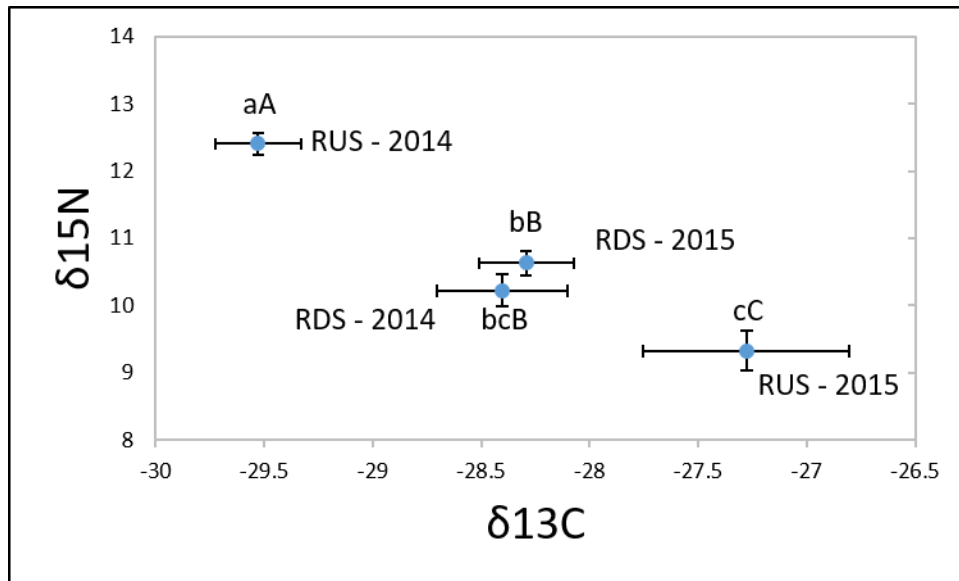


**Figure 2.3.** Stained gonadal tissues from adult male and female adult FHMs collected in Wascana Creek upstream (left; RUS) and downstream (right; RDS) of the City of Regina in 2014. Adult male testes (1) containing spermatogonia (SG), spermatocytes (SC), spermatids (ST), and spermatozoa (SZ). RDS male (1b) showing moderate (grade 3) increase in proportion of spermatogonia (SG) compared to RUS male (1a). Male upstream (2a) displaying non-remarkable degeneration, with RDS male (2b) showing a mild (grade 2) increase in testicular degeneration with losses of cell architecture, apoptotic germ cell formation and cell shrinkage and fragmentation. Ovarian tissue showing non-remarkable atresia in females upstream (3a) and a moderate (grade 3) increase in oocyte atresia in females downstream (3b). Asterisks (\*) indicate the relatively few non-atretic oocytes in the exposed female tissue. (4) Mid-development (stage 2) was observed in upstream females (4a) with vitellogenic (V) and cortical alveolar (CA) follicles, while undeveloped (stage 0) was observed in downstream females (4b) with the tissue consisting of entirely primary (P) follicles (paraffin, H&E).

### 2.4.3 Stable isotope analysis

Stable nitrogen ( $\delta^{15}\text{N}$ ) and carbon ( $\delta^{13}\text{C}$ ) isotope data obtained from spleens of fathead minnows differed significantly between sites and years (Figure 2.4). In 2014, nitrogen isotope analysis of the spleen tissues showed a significant enrichment upstream compared to the downstream site ( $p \leq 0.0005$ ). The opposite trend was seen in 2015 with the upstream site having a significantly lower signature compared to downstream ( $p < 0.01$ ). Between years we saw significant differences at RUS ( $p < 0.001$ ), but no differences at RDS ( $p = 0.481$ ).

Carbon isotope analysis for 2014 showed a significant enrichment at RDS compared to RUS ( $p \leq 0.05$ ). In 2015, the opposite trend was observed with the upstream site having a significantly lower  $^{13}\text{C}$  signature compared to downstream ( $p \leq 0.05$ ). Between years we saw significant differences at RUS ( $p \leq 0.001$ ), but no difference at RDS ( $p = 0.110$ ).



**Figure 2.4.** Stable isotope bi-plot showing nitrogen  $\delta^{15}\text{N}$  and carbon  $\delta^{13}\text{C}$  values (mean  $\pm$  SEM) for sites RUS and RDS in 2014 and 2015. Statistically significant differences between sites are denoted by lower case letters for  $\delta^{15}\text{N}$  and uppercase letters for  $\delta^{13}\text{C}$ .

#### **2.4.4 Chemical analysis**

All chemicals detected were at higher concentrations at RDS compared to RUS with the exception of clofibrate (Table 2.3). A wide variety of antibiotics, prescription and non-prescription drugs, as well as other wastewater related compounds were detected downstream of the effluent fallout. DEET, an insecticide, gemfibrozil, an antihyperlipidemic, and triclosan, an antimicrobial disinfectant, were detected at the highest concentrations of 172.2, 46.2, and 33.2 ng/L, respectively. Reproductive hormones, estradiol, estrone, progesterone and testosterone were all below the detection limits at both sites. Similarly, the synthetic ovulation inhibitor EE2 was also non-detect upstream or downstream of the effluent fallout.



**Table 2.3.** Summary of analytical results of in-stream water samples from RUS and RDS in 2014 for 22 organic wastewater contaminants. Chemical concentrations are shown in ng/L. Concentrations below the method detection limit are denoted by <MDL.

Chemical (ng/L)	Regina Upstream Site (RUS)	Regina Downstream Site (RDS)	Use
Atrazine	0.2	0.3	Herbicide
Caffeine	0.5	3.4	Stimulant
Carbamazepine	0.2	19.4	Anticonvulsant
Chlorpyrifos	<MDL	<MDL	Insecticide
Clofibrate	74.3	<MDL	Antihyperlipidemic
DEET	37.2	172.2	Insecticide
Diazepam	<MDL	0.2	Benzodiazepine
EE2	<MDL	<MDL	Ovulation inhibitor
Estradiol	<MDL	<MDL	Reproductive hormone
Estrone	<MDL	<MDL	Reproductive hormone
Gemfibrozil	<MDL	46.2	Antihyperlipidemic
Ibuprofen	<MDL	<MDL	Anti-inflammatory
Miconazole Nitrate	<MDL	<MDL	Antifungal
Naproxen	<MDL	<MDL	Anti-inflammatory
Pentachlorophenol	<MDL	4.2	Pesticide
Progesterone	<MDL	<MDL	Reproductive hormone
Sulfamethoxazole	<MDL	<MDL	Antibiotic
Testosterone	<MDL	<MDL	Reproductive hormone
Triclocarban	<MDL	0.1	Antimicrobial disinfectant
Triclosan	2.0	33.2	Antimicrobial disinfectant
Trimethoprim	<MDL	<MDL	Antibiotic

## 2.5 Discussion

The present study demonstrated that reproductive fitness of native fathead minnow populations downstream of the effluent fallout (RDS) in Wascana Creek, SK, Canada was significantly impacted. This is evidenced by changes in condition factor and organosomatic indices as well as alterations and delays in maturation of gonads of fish collected downstream compared to upstream. Effluents from WWTPs are complex mixtures whose composition may vary both seasonally and temporally (Harries et al., 1996; Alvarez et al., 2009) and as demonstrated in this study, the responses of fathead minnows at RDS exposed to these complicated mixtures also have the potential to vary.

Site-specific responses of fish with regard to condition factors varied year to year. In 2014, females were of similar size and weight both up- and down-stream of the effluent fallout, while in 2015, female fish were significantly longer and heavier and had lower condition factors downstream. Differences between years were also noted in males, with shorter and lighter fish with higher condition factors in 2014 downstream, but no significant differences in size, weight or condition observed in 2015 (Table 2.2). The minimal changes in condition factors observed in this study are similar to findings in which no changes, or similar energy allocations were observed in downstream roach (Jobling et al., 1998), flounder (*Platichthys flesus*; Allen et al., 2009) and white sucker (*Catostomus commersoni*; Hinck et al., 2009) compared to un-exposed fish. In other studies with wild fish exposed to MWWs, varying responses have been reported in respect to condition factor. Increases have been observed in many fish species, including longnose suckers (*Catostomus catostomus*) and longear sunfish (*L. megalotis*) likely due to increases in nutrients released within the treated effluents (Adams et al., 1992; McMaster et al., 2005; Porter and Janz, 2003). This hypothesis may explain the relatively high condition factor observed in male FHMs

at RDS in 2014 however, it does not explain the differences seen between sexes. Alternatively, decreases in fish condition have been observed in male feral carp (*Cyprinus carpio*), hypothesized to be due to high estrogenic exposure having a significant impact on the physiological state of the fish affecting this health parameter (Sole et al., 2002). However, this hypothesis likely does not explain the decreased condition observed in female FHMs at RDS in 2015, based on the absence of estrogenic compounds in our analytical findings.

In both sexes for 2014 and 2015, significant increases in hepatosomatic index were observed at RDS (Table 2.2). HSI provides an indication of energy status, and therefore, an increase in liver size could be a sign of increased metabolic activity due to contaminant exposure and overall increase in energy storage (EVS, 1999). Hypertrophy of the liver due to the removal of toxicants has been reported as a hypothesis for increases in liver size with exposure to MWWs (Porter and Janz, 2003; Yeom et al., 2007). Previous studies have also shown that higher HSIs, indicating increased energy stores, may be due to the elevation of nutrients associated with MWWs, and not increased enzyme activity, in response to changes in lipid or glycogen storage and food supply (Munkittrick et al., 1994; Porter and Janz, 2003; McMaster et al., 2005; Tetreault et al., 2011). Additionally, a fish liver, specifically females, is comprised of many estrogen receptors allowing them to produce great amounts of vitellogenin when stimulated by an estrogen (Sumpter and Jobling, 1995). Elevated HSI values have been associated with increases in vitellogenin production in response to estrogen exposure (Carragher and Sumpter, 1991). With exposure to estrogenic compounds, elevated HSIs correlated with increases in VTG production have been widely reported (Janssen et al., 1995; Diniz et al., 2005; Werner et al., 2003). In this study, the increase in liver sizes of fish collected downstream of the effluent fallout are likely attributed to the hypothesis of induction of liver detoxification enzymes with exposure to toxic

contaminants associated with effluents from the City of Regina causing increases in the liver sizes, and not due to vitellogenin induction as some studies have reported, because of the consistent absence of estrogenic effects in our endpoints as well as the lack of detection of estrogenic compounds in the surface water. Similar to Waiser et al. (2011), the increases observed here may also be influenced by the elevated concentrations of both nitrogen (N) and phosphorus (P) downstream of the effluent fallout in July of 2014 and 2015 (Table 2.1). However, changes in lipid or glycogen storage and food supply were not assessed in this study, and therefore, further investigation into liver enzymatic activity and energy stores would help to determine this relationship.

GSI of both males and females at RDS were at least 2x lower in both 2014 and 2015 compared to upstream populations (Table 2.2). GSI values provide an indication of the sexual maturity of fishes, and have been widely used as a good indicator of exposure to compounds causing deleterious effects on reproduction, specifically EDCs. Reductions in GSI of fish exposed to MWWs, often containing many EDCs, has been widely reported in a variety of species of fish with effects related to inhibition of maturation (bream: Hecker et al., 2002; mirror carp: Diniz et al., 2005; white sucker: Vajda et al., 2008; greenside darters: Tetreault et al., 2011), metabolic disruption (Munkittrick et al., 2000, 2002), and decreased hypothalamic, pituitary or gonadal activity (fathead minnows: Hachfi et al., 2012). Alternatively, increases in GSI of fish exposed to industrial and municipal effluents have also been previously reported and associated with increased nutrients or food supply (McMaster et al., 2005; Munkittrick et al., 2002) or reabsorption of gonadal material due to delayed spawning (Tetreault et al., 2011). While Hachfi et al. (2012) stated that typically GSI values are reduced after exposure to estrogenic or antiandrogenic contaminants, in this study, it is likely the effects observed are due to a number of different

stressors leading to delayed or inhibited maturation of gonadal tissues. These potentially include exposure to high nutrient levels, a number of different EDCs found in the effluents, and most importantly, the high concentrations of ammonia in the effluents released into the system during the sampling period. While not necessarily endocrine specific, elevated ammonia levels have been shown to negatively impact reproduction (Armstrong et al., 2012) at levels as low as 0.06 mg/L (Armstrong et al., 2012), significantly lower than the average concentrations observed in the City of Regina effluents of 0.241 and 0.102 mg/L for July 2014 and 2015 respectively. GSI values can provide indications of impacts on gonadal development and maturity, with lower GSI values most often being linked to pathological alterations in the gonads (Kinnberg et al., 2000). This is discussed further below in histopathology of gonads.

Lower secondary sexual characteristic scores observed in males at RDS compared to RUS for both years suggest demasculinization of males exposed to MWWs as development of these characteristics are androgen dependent. For example, induction of nuptial tubercles and a fatpad in males and females has been observed with exposure to androgens (Harries et al., 2000). Alternatively, losses of nuptial tubercles and dorsal fatpad formation in male fathead minnows have been associated with exposure to wastewater effluents (Vajda et al., 2011) and its common constituent's 17 $\beta$ -estradiol (Miles-Richardson et al., 1999) and fluoxetine (Shultz et al., 2011). As secondary sexual characteristics play a key role in fathead minnow spawning behaviours (Smith, 1978) their disruption may adversely impact the fitness of the fish in territorial contests, access to mates or quality of parental care (Grist et al., 2003; Lange et al., 2011 and Scott and Sloman, 2004).

In addition to effects on somatic indices and secondary sex characteristics, there were significant alterations in gonadal histopathology between the up- and downstream site. The

significant increase in proportions of spermatogonia and decrease in spermatocytes in males at RDS were similar to findings from earlier studies by Tetreault et al. (2011) and Kidd et al. (2007) with wastewater- and EE2-exposed male fathead minnows, respectively. This shift in cell type between the up- and downstream site suggests a delay in gonadal maturation of male fish at RDS. Similar delays in spermatogenesis have been well documented in wild fish exposed to treated sewage effluent (Diniz et al., 2005; Douxfils et al., 2007). Less mature testes have also been observed with exposure to the anti-androgen, flutamide (Jensen et al., 2004). In this study, reproductive capabilities of RDS males were likely to have been further affected in downstream fish due to increased testicular degeneration that was observed in the same fish (Figure 2.2). General degeneration of testicular tissue has been reported in fish exposed to treated sewage effluent (Diniz et al., 2005) and to the xenoestrogen EE2 (Kidd et al., 2007). More specifically, loss of germ cells, or the formation of germ cell syncytia has been observed in male fathead minnows exposed to 17 $\beta$ -estradiol (Miles-Richardson et al., 1999).

One condition that has been highly documented in fish exposed to MWW (darters: Tetreault et al., 2011; roach: Bjerregaard et al., 2006; mirror carp: Diniz et al., 2005; roach: Jobling et al., 1998; medaka: Zha et al., 2006) and some of its common constituents such as EE2 (fathead minnow: Laenge et al., 2001; Kidd et al., 2007), 17 $\beta$ -estradiol (fathead minnow: Patino and Redding, 2000), 4-*tert*-pentylphenol (fathead minnow: Panter et al., 2006), and pentachlorophenol is mixed sex or intersex (female oocytes in male testicular tissue). Interestingly, there was no evidence of the occurrence of testicular oocytes or mixed sex conditions in males collected from RDS, suggesting that populations downstream were not exposed to estrogenic compounds, like those mentioned above, at concentrations great enough to cause this phenomenon. This is further supported with the lack of detection of reproductive hormones or hormone agonists (e.g. estradiol,

testosterone, estrone, estriol, EE2) in the surface water samples analyzed at RDS. However, the failure to allocate energy towards gonadal development (lower GSI and delayed spermatogenesis) and general degeneration of the tissues resulted in reproductive impairment in this study.

This study also demonstrated significant histological alterations in females at RDS. Females downstream were less mature with smaller gonads, significantly fewer vitellogenic follicles and increase atresia of oocytes (Figure 2.2). Suppression of ovarian development in fathead minnows has been observed for a variety of endocrine active compounds such as the androgen methyl dihydrotestosterone (Bogers et al., 2006), the pesticide vinclozolin (Makye et al., 2000) and the estrogen 17 $\beta$ -estradiol (Miles-Richardson et al., 1999). Furthermore, similar degradation of reproductive tissues has been previously reported in fathead minnows after exposure to estrogens (Wolf et al., 2004), androgens (Ankley et al., 2001; Pawlowski et al., 2004), anti-androgens (Jensen et al., 2004), p450 inhibitors (Ankley et al., 2002), industrial chemicals (Ankley et al., 2001), and pesticides (Ankley et al., 2005), all of which are compounds potentially found in MWWs. Also, inhibition of maturation and oocyte degeneration have been observed in fish downstream of WWTPs and urban areas (Doux fils et al., 2007; Diniz et al., 2005; Hecker et al., 2002). Similar histological alterations were observed in both male and female FHMs exposed to 50% Regina MWW in a parallel study (Steeves, 2018), which confirmed that MWW was likely responsible for the observed effects. Overall, this study demonstrated major alterations in the histopathology of both male and female fathead minnow gonadal development downstream of the City of Regina WWTP, and which indicated likely impacts on the reproductive fitness of these animals.

Analysis of stable isotopes in this study confirmed site fidelity at RDS for both 2014 and 2015, with no differences in  $\delta^{15}\text{N}$  and carbon  $\delta^{13}\text{C}$  signatures (Figure 2.4). The increased  $\delta^{15}\text{N}$

values in tissues of RDS fish downstream of the effluent fallout in 2015 are similar to previous studies reporting that increased human inputs of nitrogen led to increasing nitrogen content values in aquatic organisms (Dube et al., 2005; Loomer et al., 2008). Interestingly, a shift in  $\delta^{15}\text{N}$  and carbon  $\delta^{13}\text{C}$  signatures was observed at RUS, suggesting differences in either site fidelity or contributions of nutrients between years (Figure 2.4). RUS is located on a section of Wascana Creek with no municipal or industrial inputs; however, it is surrounded by agricultural land, where runoff may occur. Rainfall was dramatically different in Regina, SK between 2014 (281.4mm) and 2015 (96.1mm) from first day of spring (March 20) to beginning of sampling (July 20) of each year. During the spring period, fertilizer is being applied to surrounding crops in the RUS area, and with the larger amount of rainfall in 2014, greater inputs of nitrogen through runoff into the creek may explain this shift in signatures at RUS between years. Increased variability in isotope signatures during spring collections has also been demonstrated by Loomer et al. (2008). The similarities between sampling years at RDS may further support that MWWs dominate this portion of the stream, and thus other factors, such as fertilizer runoff, do not seem to influence as much as at sites that lack the effluent input.

Of the 22 compounds analyzed, only 10 PPCP's and their metabolites were detected above the method detection limit in the ng/L to  $\mu\text{g/L}$  range downstream of the effluent fallout. There were five categories of use for chemicals found in surface water collected at RDS, pesticides (atrazine, DEET, and pentachlorophenol), a stimulant (caffeine), antimicrobials (triclocarban and triclosan), anti-convulsant (carbamazepine), and lipid regulators (gemfibrozil) (Table 2.3). While Waiser et al. (2011) detected the presence of 25 PPCP's and their metabolites downstream of the City of Regina WWTP in water surveys throughout the year, only 13 were detected at comparable sampling time and location (July 2006, Site 2) to this study. Compounds detected varied between



studies, but carbamazepine, DEET, gemfibrozil and triclosan were consistently found. However, concentrations for these compounds detected at RDS in this study were all lower, with the exception of DEET, compared to Waiser et al (2011) findings. Interestingly, concentrations of hormones, estrone (E1), estradiol (E2) and EE2, were all below the detection limit at RDS and well below the median concentrations in sewage effluents of 19, 5.5, and 9.00 ng/L, respectively, reported across Canada (Sun et al., 2014). Additionally, in a parallel study, Bagatim (2019), found Regina effluents not to be significantly estrogenic using an *in vitro* assay (MVLN bioassay) in spring 2014. In this study, chemical analysis was only conducted during the sampling period of 2014, but it has been shown in previous studies that concentrations vary throughout the year, with the highest concentrations occurring most often during winter months (Waiser et al., 2011). This allows for changes in exposure of aquatic organisms throughout the year. In general, the chemicals detected at RDS are similar to those reported in other studies, and concentrations found here are within the lower range of contaminant reporting.

Of the 10 chemicals that were detected at RDS, DEET (172.2 ng/L), gemfibrozil (46.2 ng/L), triclosan (33.2 ng/L) and carbamazepine (19.4 ng/L) had the greatest concentrations. DEET, an insect repellent, has a relatively low toxicity with LC50's for aquatic organisms being greater than 100 mg/L (Waiser et al., 2011) and chronic NOECs for daphnids and green algae ranging from 0.5 to 24 mg/L (Weeks et al., 2011), however DEET has been shown to have anti-androgenic activity in female FHMs exposed to 600 ng/L (Zenobio et al., 2014). DEET was also present at RUS in lower concentration (37.2 ng/L) and is likely present due to the proximity to a local golf course. Gemfibrozil is found ubiquitously in surface water systems with concentrations in the ng/L range in Europe and North America (Fent et al., 2006; Waiser et al., 2011; Quinn et al., 2008). Gemfibrozil concentrations found at RDS were relatively high (46.2 ng/L), when

compared to concentrations previously reported in undiluted MWWs in Canada, ranging between 59 ng/L in Montreal, QB, and 246 ng/L in the Thames River, ON (Lishman et al., 2006), and concentrations measured across Europe were between 60 ng/L in France and 0.71 ng/L in Greece (Quinn et al., 2008). While exposure to gemfibrozil at low concentrations (1.5 µg/L) has been shown to reduce levels of testosterone in goldfish (Mimeault et al., 2005), and induce proinflammatory cytokines on a marine fish (Teles et al., 2016), it is unlikely that any effects observed in RDS fish are due to gemfibrozil exposure simply due to much lower exposure concentration (46.2ng/L). Triclosan is found in MWW receiving surface systems around the world, with concentrations in effluents in Europe and North America ranging from 0.1 to 2.7 µg/L (Samsøe-Petersen et al., 2003). Triclosan has been reported to have endocrine disrupting properties with increases in VTG production and HSI and decreases in sperm counts in male mosquitofish at similar concentrations to what was observed in this study (Raut and Angus, 2010). The increases in HSI and decreases in sexual maturity observed in RDS males therefore may be explained by exposure to triclosan at these levels (28.9 ng/L). Toxic effects of triclosan have also been observed at relatively low concentrations on algae (1.4 µg/L; Yang et al., 2008), and hazard quotients generated by Waiser et al., (2011) and the concentrations reported in this study (33.2 ng/L) suggest triclosan may present a potential risk to both algae and benthic invertebrates in Wascana Creek as well. Carbamazepine is consistently found in MWWs worldwide and while concentrations in this study were relatively low (0.019 µg/L), concentrations of up to 2.3 µg/L have been reported in Canada (Metcalf et al., 2003b) and up to 6.3 µg/L in Germany (Ternes 1998). Endocrine effects such as decreased androgen levels and reduced fish reproduction have been observed with chronic exposure of fish to carbamazepine at 10,000 ng/L, much higher than concentrations detected (Fraz et al., 2018). Due to the relatively low toxicity of DEET and

caffeine, the concentrations measured at RDS likely do not explain the effects observed. However, the presence of gemfibrozil and triclosan at greater concentrations may have contributed to the effects we observed. Ultimately, while many of these compounds are present at low concentrations individually in Wascana Creek, SK, they exist in a complex mixture, with the potential for cumulative effects and increased toxicity to aquatic organisms. Therefore, further investigation for pharmaceutical mixtures is needed.

The concentrations ranging from 0.066 to 0.511 mg/L of un-ionized ammonia ( $\text{NH}_3\text{-N}$ ) in the final effluents released just upstream of RDS at the time of our sampling also raised significant concerns as exposure to un-ionized ammonia at levels as low as 0.06mg/L have been shown to increase mortality, reduce growth and impair reproductive output in fathead minnows with a 20-day exposure (Armstrong et al., 2012). Additionally, Waiser et al. (2010) measured alarmingly high concentrations of total ammonia downstream of the effluent fallout in Wascana Creek, far exceeding both the Canadian and American water quality guidelines with levels of un-ionized ammonia likely high enough to cause toxicity to aquatic organisms such as fish, mollusks, amphipods and planarians throughout the year. Furthermore, Steeves (2018) found un-ionized ammonia levels around 0.6 mg/L in 50% diluted effluents collected during the same sampling year as this study (2014) from the Regina WWTP. The elevated ammonia levels present in the creek, may allow fathead minnow populations at RDS to have a net uptake of ammonia, potentially causing some of the adverse biological effects we observed. Sub-lethal effects in fathead minnows with ammonia exposure include growth reductions (Fairchild et al., 2005), inhibited reproductive success (Thurston et al., 1986; Armstrong et al., 2012), kidney malformations ((Tetreault et al., 2011), irregular gill ventilation (Tetreault et al., 2011), and decreased brain monoamines (Ronan et al., 2007).

## 2.6 Conclusions

MWWEs released into Wascana Creek, SK from of the City of Regina's WWTP, caused adverse biological effects such as altered condition, increased liver sizes, delays in maturation, and degradation of reproductive tissues in resident fathead minnow populations, all of which are likely to weaken their ability to reproduce and survive. Stable isotope analysis provided insight into site fidelity and the ability of the RDS fish to be self-sustaining. Interestingly, typical responses to the exposure with estrogens such as mixed sex condition that have been previously hypothesized to be one of the main concerns for male fish downstream of MWWEs were not observed. This lack of estrogenicity is supported by the absence of estrogenic compounds in water chemistry samples at RDS and with non-significant estrogenicity reported in a parallel *in vitro* study (Bagatim, 2019). Alternatively, Regina effluents showed significant anti-androgenic and anti-estrogenic activity (Bagatim, 2019), which is in accordance with the impacts on reproductive parameters observed in this study. Ultimately, overall toxicity and suppression of reproductive systems in the exposed populations was likely occurring due to a culmination of a diverse number of stressors including high nutrient and ammonia levels and presence of wide ranging PPCPs.

Future work using the same samples will include gene expression analysis in the brain, liver and gonad tissues and changes in plasma hormone levels of both upstream and downstream fathead minnows in 2014 and 2015 to provide insight into potential mechanisms for the reproductive effects observed here. Together these studies will provide a clearer understanding of the potential impacts of MWWEs on fathead minnow populations inhabiting the effluent dominated stream Wascana Creek, SK as well as risks associated across Canada and the need for implementing more effective and efficient treatments to remove emerging contaminants.

Furthermore, future studies should be conducted to investigate if the upgrades completed at the Regina WWTP help to improve the overall quality of Wascana Creek and health of its aquatic life.

## **Acknowledgements**

The authors would like to thank A. Moate, D. Gagnon, B. Eisner, J. Doering, S. Beitel, A. Masse and all other staff and students who provided laboratory and/or field assistance throughout this study. Special thanks to the City of Regina Wastewater Treatment plant for their cooperation. This project was supported through the Canada Research Chair program and the Canadian Water Network. M. Hecker was supported through the Canada Research Chairs program.

**Chapter 3 : MOLECULAR BIOMARKERS AS MECHANISTIC  
EVIDENCE OF MUNICIPAL WASTEWATER EFFLUENT  
EFFECTS ON WILD FATHEAD MINNOW (*PIMEPHALES  
PROMELAS*) POPULATIONS IN AN EFFLUENT DOMINATED  
STREAM, WASCANA CREEK, SK, CANADA.**

## PREFACE

This chapter was developed based on the results of Chapter 2, where the health and reproductive status of wild fathead minnow populations inhabiting an effluent dominated system, Wascana Creek, Saskatchewan, Canada was determined. From Chapter 2, adverse biological effects were observed in fish downstream of the City of Regina WWTP; however, insight into the molecular drivers behind the apical endpoints were not determined. Therefore, Chapter 3 further evaluates the potential mechanisms for the reproductive effects observed in Chapter 2. This chapter was organized as a manuscript for publication in a peer-reviewed scientific journal.

### Author Contributions:

Sara Hanson (University of Saskatchewan) designed and managed the experiment, generated and analyzed all data, and drafted the manuscript.

Tabata Bagatim (University of Saskatchewan) conducted a parallel study on the endocrine potentials municipal effluents from two prairie WWTPs, providing information on *in vitro* studies to correlate with *in vivo* results.

Kean Steeves (University of Saskatchewan) conducted a parallel study on the endocrine system of laboratory fish exposed to municipal effluent from two prairie WWTPs, providing information on laboratory *in vivo* studies to correlate with field based *in vivo* results.

Steve Wiseman (University of Lethbridge) provided guidance throughout experiments and offered comments and edits to the manuscript.

Natacha Hogan (University of Saskatchewan) provided guidance throughout *in vitro* experiments and offered comments and edits to the manuscript.

Alice Hontela (University of Lethbridge) provided guidance throughout *in vitro* experiments, offered comments and edits to the manuscript.

Paul Jones (University of Saskatchewan) provided guidance throughout chemical analysis experiments, as well as offered comments and edits to the manuscript.

John Giesy (University of Saskatchewan) provided the molecular laboratory to conduct the mechanistic portion of study and offered comments and edits to the manuscript.

Markus Hecker (University of Saskatchewan) provided guidance and inspiration for the conception and design of the experiment, offered comments and edits to the manuscript, and provided research funding.



### 3.1 Abstract

The effects of municipal wastewater effluents (MWWEs) on local aquatic eco-systems in semi-arid regions, such as the Canadian prairies, are limited, and concerns surround the occurrence of emerging contaminants and their potential to adversely impact resident organisms. In particular, there is significant uncertainty regarding the mechanisms by which MWWEs elicit their effects on reproduction and health in wild fish populations. This project aimed to characterize the specific molecular mechanisms driving biological alterations, including delayed maturation and gonadal alterations, previously observed in wild fathead minnow (*Pimephales promelas*; FHM) populations collected from an effluent dominated stream, Wascana Creek, SK. To better understand the cause for these disruptions and potentially distinguish specific components of these complex mixtures responsible for the effect observed, field studies were conducted on spawning FHMs (2014 and 2015) to measure circulating sex steroid hormone concentrations and expression of key genes along the hypothalamus-pituitary-gonadal-liver (HPGL) axis. While not statistically significant, females and males downstream of the City of Regina's wastewater treatment plant (WWTP) showed a clear trend towards increased plasma estradiol and decreased plasma 11-ketotestosterone levels, respectively, compared to their upstream counterparts in both 2014 and 2015. Key biomarkers of estrogenic exposure, such as induction of vitellogenin (VTG) was not observed in exposed male FHMs. Lowered expression of estrogen ( $ER\alpha$ ,  $ER\beta$ , VTG) and androgen responsive genes (AR) in populations downstream suggest that estrogenic compounds likely did not cause the effects observed in the parallel study. In exposed fish downstream of the WWTP there was evidence of down-regulation of key steroidogenic genes, CYP19 $\beta$  and StAR, coupled with lowered FSHR and LHR expression, suggesting that steroidogenic capacity in the gonads of fish exposed to the MWWEs had been reduced. The results of this study demonstrated that

exposure to MWWWE can impact the overall health and reproductive status of exposed wild fish populations, however, these effects do not appear to be due to estrogenic or androgenic compounds in the effluent, which is in accordance with the lack of feminization of males or masculinization of females reported in a parallel study. Furthermore, the general inhibition of reproductive functions of FHMs downstream of the City of Regina's WWTP are in accordance with two other studies investigating the effects of these effluents on a 21 day reproductive assay and *in vitro* bioassay studies evaluating the endocrine potentials of the effluents.

### **3.2 Introduction**

There is increasing public concern surrounding the occurrence of emerging contaminants (ECs) in aquatic ecosystems and their potential to adversely impact resident organisms (Kolpin et al., 2004). ECs are a diverse class of compounds, and include personal care products and pharmaceuticals (PPCPs), soaps and detergents, plasticizers, pesticides as well as hormones and chemicals released from livestock operations, among others (Purdum et al., 1994; Folmar et al., 1996; Hoffman and Oris., 2006). These compounds are found in agricultural runoff, industrial effluents and municipal wastewater effluents (MWWEs), with the latter representing one of the most prominent sources of ECs (CCME, 2006). MWWEs are complex mixtures of organic waste, nutrients, and pathogens, as well as a suite of ECs such as steroidal estrogens, PPCPs and household and industrial chemicals that are not or are only partially removed during the wastewater treatment process (Desbrow et al., 1998; Mills and Chichester, 2005; Baronti et al., 2000).

ECs are typically found in effluents, receiving waters, sediments and tissues of exposed organisms at concentrations below acute toxicity (Vidal-Dorsch et al., 2011; Maruya et al., 2011); however, even very low concentrations of some ECs have been shown to cause significant

sublethal effects, including endocrine disruption, affecting growth, development and reproduction of aquatic organisms, particularly fish (Campbell et al. 2006; Hecker et al., 2006; Goksøyr 2006). Furthermore, while concentrations of ECs are generally low in MWWs, risks to the environment cannot be excluded due to the continuous release of these compounds resulting in pseudo-persistence. These risks are amplified in semi-arid environments, like the Canadian prairies, where receiving systems have low flow and are quite small resulting in minimal dilution of the effluents (Cwiertny et al., 2014; Dotan et al., 2016; Waiser et al., 2011). Additionally, these prairie ecosystems are characterized by an extreme temperature regime (+45°C to -50°C), potentially impacting the efficiency of biological treatment processes at lagoon based treatment plants that are still common practice in this region, resulting in greater loading of contaminants such as ECs and nutrients into the effluents. However, even though these potential risks have been identified, minimal research has been done in the Canadian prairies in regards to the impacts of MWWs (Waiser et al., 2011). Furthermore, information is limited on the effects of MWWs on local aquatic eco-systems in other semi-arid regions of the world including the central United States, Asia, Eastern Europe and sections of South America. Because of their similarities, inferences can likely be made between different cold semi-arid regions around the world with regards to the environmental impacts of MWWs on receiving systems and associated organisms.

One type of biological effect that has been shown in fish downstream of many waste water treatment plants (WWTPs) is disruption of normal reproductive functioning due to exposure of endocrine disrupting compounds (EDCs) found in MWWs and their associated ECs. While this phenomenon has been widely evaluated on wild fish species in both Europe and North American freshwater environments, only little is known about similar impacts in prairie ecosystems. In particular, estrogenic impacts in male fish downstream of WWTPs, resulting in the presence of

female germ cells in the testes, have been among one of the most frequently reported phenomena (Purdom et al., 1994; Folmar et al., 1996; Kidd et al., 2007). In addition to this intersex condition, exposure of fish to MWWEs has resulted in other reproductive impairments such as delayed gonad maturation, altered gonadal development, altered secondary sexual characteristics and changes in organosomatic indices (Chapter 2; Ankley et al., 2003; Diniz et al., 2005; Hecker et al., 2002; Tetreault et al., 2011). While physiological and toxicological responses such as those listed above are often the ones assessed in regulatory processes, responses can vary due to different interactions of diverse chemicals found in MWE mixtures and their varying molecular processes. Therefore, it is important to characterize specific molecular mechanisms driving these biological alterations to not only better understand the cause for these disruptions and distinguish specific components of these complex mixtures responsible for the effects observed, but to also potentially develop predictive mechanistic toxicity pathway models for characterization of effluent toxicity in fish, provided that molecular effects are indicative of apical outcomes. The latter would ultimately reduce the need for extensive live animal testing, including wild fish studies such as this, by conducting *in vitro* or short-term *in vivo* tests predicting these apical outcomes.

Endocrine disrupting compounds (EDCs), including many ECs found in MWWES, are able to cause a wide variety of effects due to their ability to act through multiple mechanisms of action. In the literature, focus has been on EDCs that mimic hormones resulting in agonistic effects on cellular processes, or alternatively that act antagonistically binding to and blocking the receptor binding site, therefore blocking the transcriptional activation triggered through these receptors (Arukwe, 2001). The activity of these receptors, such as estrogen receptor (ER) and androgen receptor (AR) can be altered if changes occur in the expression levels of these receptors and/or co-regulator mRNAs and proteins (Tabb and Blumberg, 2006). Due to the regulation of the induction

of vitellogenin (VTG), an egg-yolk precursor protein, by the estrogen receptor, induction of VTG is indicative of estrogenic stimulation, making it an excellent biomarker of exposure to estrogenic chemicals and complex mixtures, such as municipal effluents. However, in addition to acting on specific receptors, EDCs can elicit effects through modulation of sex steroid hormone production, or steroidogenesis (Craig et al., 2011). Steroidogenesis is a biosynthesis pathway involving a series of steps converting cholesterol into sex steroid hormones such as estradiol (E2), testosterone (T) and 11-ketotestosterone (11KT), the most active reproductive androgen specific to fish, and is controlled by the hypothalamus-pituitary-gonadal-liver (HPGL) axis (Arukwe, 2008; Hogan et al., 2010). This pathway is tightly regulated by feedback loops that up- and down-regulate development, growth and reproductive processes. Disruption of steroidogenesis can alter the transcription, expression or activity of enzymes and target genes involved in this process. This can result in alterations in the homeostasis of sex steroid concentrations, which ultimately leads to disruption of the reproductive endocrine system and the process of reproduction (Ankley and Johnson, 2004). Additionally, changes in sex steroid hormones have been shown to impact other key systems involved in immune function, brain development and behaviour (Arcand and Benson, 1997, Hecker et al., 2006). Therefore, disruption of the activity and expression of genes involved along the HPGL axis and/or circulating levels of these hormones is likely to greatly impact sexual development, reproduction, growth and overall health status.

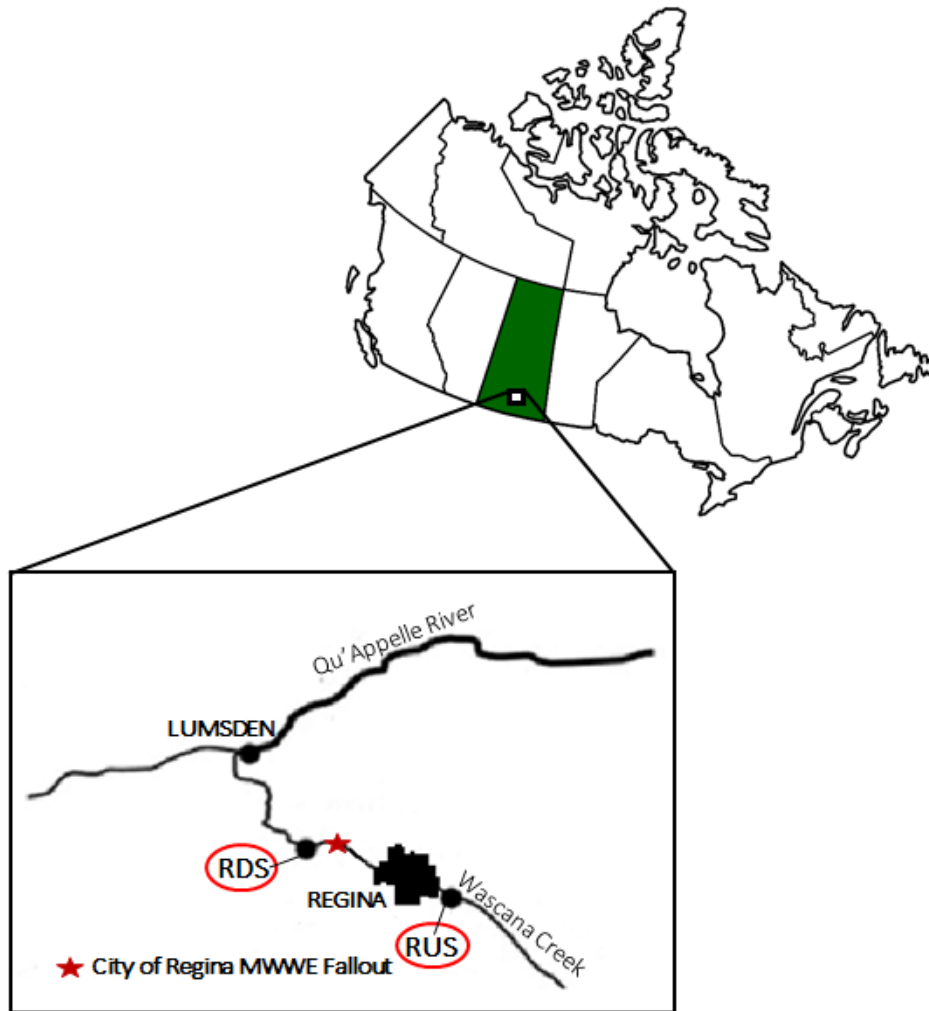
The overall aim of this present study was to investigate endocrine function-related molecular changes in wild fathead minnow populations in a small prairie stream, Wascana Creek, SK, Canada exposed to MWWs. Wascana Creek was selected as a representative high risk system, as it is an effluent-dominated stream receiving MWWs from the City of Regina's outdated lagoon based treatment facility, is small in size and is located in a semi-arid cold prairie

environment. The creek receives effluents at a rate of  $0.90\text{m}^3/\text{s}$ , and in winter, the treated sewage effluent can make up to 99% of the creek flow until it connects with the Qu'Appelle River 60km downstream (Waiser et al., 2011). Specifically, the present study aimed to characterize the specific molecular mechanisms by which effluents released by the Regina WWTP affect resident populations of fathead minnows up- and downstream of the WWTP outfall by 1) characterizing changes in plasma sex steroid hormone levels; 2) describing alterations in abundances of key genes along the hypothalamus-gonad-liver axis; and 3) comparing molecular changes measured to apical outcomes in the same fish described in a parallel study to identify possible molecular drivers of toxicity (Chapter 2). In the parallel study (Chapter 2), adverse biological effects such as altered condition, increased liver sizes, delays in maturation and degradation of reproductive tissues in FHM's downstream of the City of Regina's WWTP were observed.

### **3.3 Materials and Methods**

#### **3.3.1 Field Sampling and tissue collection**

Adult fathead minnows were collected during spawning season (late July) in 2014 and 2015 from two locations in Wascana Creek, SK, Canada; a reference site upstream of the city (RUS; 50.40, -104.49), and directly downstream of the effluent fallout (RDS; 50.48, -104.75) as described previously (Chapter 2). Seine nets were pulled from shore to shore and fathead minnows collected were removed and transported to an on-site mobile laboratory in aerated pails.



**Figure 3.1.** Site locations for sampling of Fathead Minnows in Wascana Creek. RUS (Regina Upstream Site); RDS (Regina Downstream Site); MWW (Municipal wastewater effluent).

Immediately following sampling, fish were anesthetized in Aquacalm (5-10 mg/L; Syndel Laboratories, Nanaimo, BC, Canada). Blood was collected from the caudal vein/artery with a heparinized 29 gauge needle and syringe. Blood was stored on ice in microcentrifuge tubes. Plasma was separated via centrifugation (6000 rpm for 5 min), transferred to Eppendorf tubes and frozen at -80 °C until steroid hormone analysis. Fish were then euthanized via spinal severance. Liver, gonads and brain tissues were excised, weighed and flash frozen in liquid nitrogen and stored at -80 °C until gene expression analysis. Phenotypic sex was determined and recorded during dissection.

### **3.3.2 Plasma hormone analysis**

Two sex steroid hormones, 17 $\beta$ -estradiol (E2) and 11-ketotestosterone were quantified using enzyme-linked immunosorbent assays (ELISA) purchased from Cayman Chemical (Ann Arbor, MI, USA). As plasma volume was limited, only E2 was measured for females and 11-KT for males. Extractions were done using a liquid-liquid extraction method in accordance with Chang et al. (2009) with minor alterations detailed in Beitel et al. (2014). Briefly, samples were extracted twice with 2 mL of a 1:1 Hexane:Ethyl Acetate mixture by vortexing the sample mixture for one minute, followed by centrifugation at 2000rpm for three minutes. The supernatant was collected, dried under a stream of nitrogen and brought up into a buffer provided by the manufacture totalling the required volume (250  $\mu$ L) for quantification. Quantification of steroid hormones was conducted in triplicate following protocols provided by the manufacturer (Cayman Chemical). Steroid hormone concentrations were expressed as ng per mL (ng/mL).



### **3.3.3 Quantitative real-time polymerase chain reaction**

Total RNA was extracted from approximately 20 mg each of liver, gonad and brain tissue using QIAGEN's RNeasy Mini Kit as described by the manufacturer (Qiagen, Mississauga, ON, Canada). The concentrations of RNA in each sample were determined using a NanoDrop ND-1000 Spectrophotometer (Nanodrop Technologies, Welmington, DE, USA). Samples of RNA were stored at -80 °C until reverse transcription was performed using 1 µg of RNA from each sample using a Quanti-TECT Reverse Transcription Kit (Qiagen) according to the protocol recommended by the manufacturer to obtain cDNA for real-time PCR. Samples of cDNA were stored at -20°C until analysis.

Quantitative real-time PCR (qPCR) was performed using 96-well PCR plates on an ABI 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). A 50 µL master mix consisting of 2.5 µL of cDNA, 2.5 µL of sense/antisense gene-specific primer, 25 µL 2x QuantiFast SYBR Green Master Mix (Qiagen), and 20 µL of RNase-free water (Qiagen) was prepared for each sample of cDNA and primer combination. Reactions were all run in duplicate with 20 µL per well. The reaction mixture for PCR was denatured at 95 °C for 10 min followed by a thermal cycle profile consisting of denaturing at 95 °C for 10 s and extension for 1 min at 60 °C for a total of 40 PCR cycles. Transcript abundances of target genes were quantified by normalizing to 18s according to the method of Simon (2003). Fourteen genes involved in reproduction pathways and processes along the hypothalamus-pituitary-gonadal-liver (HPGL) axis were selected. Genes and nucleotide primer sequences were designed based on the sequences available from He, et al., 2012 and available in the NCBI GeneBank database and were ordered from Invitrogen (Burlington, ON, Canada; Table 3.1).

**Table 3.1.** Nucleotide sequence, efficiencies and GenBank accession numbers of primer pairs used in qPCR for brain, gonad and liver tissues. Sequences from He., et al 2012.

Target Gene	Primer	Sequence (5'-3')	Tissue	Efficiency	GeneBank
d17 $\beta$ -hydroxysteroid dehydrogenase (17 $\beta$ HSD)	Forward	ATCCAGAGTGTGCTGCCTTT	Gonad	98%	DT161033
	Reverse	AGGGAAATAGCCGTTGGTCT			
Androgen Receptor (AR)	Forward	CAACGCGTCTAAATCCCAT	Brain, liver	93, 95%	AY727529
	Reverse	TGTTCGAACTGACACGAAGC			
Aromatase $\alpha$ (CYP19 $\alpha$ )	Forward	GCTGCACAAGAAGCACAAAG	Gonad	94%	AF288755
	Reverse	CGTGGCTCTGAGCGAATATC			
Aromatase $\beta$ (CYP19 $\beta$ )	Forward	AGGGTGTATCCTGGCAACTG	Brain	98%	AJ277866
	Reverse	ATCTGCACCCGTTTCATTTTC			
Estrogen Receptor $\alpha$ (ER $\alpha$ )	Forward	CGGTGTGCAGTGACTATGCT	Brain, liver	94, 84%	AY775183
	Reverse	CTCTTCCTGCGGTTTCTGTC			
Estrogen Receptor $\beta$ (ER $\beta$ )	Forward	CGTTTTGGCATAACCATGTG	Brain, liver	105, 96%	AY566178
	Reverse	TGCTGTGAGACTTCCGAATG			
Follicle-stimulating hormone receptor (FSHR)	Forward	CACGTAAGTGTGTCAGACG	Gonad	90%	EF219401
	Reverse	GTGGCTGGGGTATGTCAGAT			
luteinizing hormone receptor (LHR)	Forward	CTTCAACCACCTTCCCAAG	Gonad	105%	DT281016
	Reverse	AGCATTTGGTGGGACTGAAC			
ribosomal RNA 18S (18S)	Forward	GCCCTGTAATTGGAATGAGC	Brain, liver, gonad	97%	AY855349
	Reverse	TCCCGAGATCCAACCTACGAG			
Steroidogenic acute regulatory protein (StAR)	Forward	ATGCCCCGAGAAGAAAGGATT	Gonad	80%	DQ360497
	Reverse	CCCGGTTGATGACTGTTTTT			
Vitellogenin (VTG)	Forward	TTGCTCTCCAGACCTTTGCT	Liver	98%	AF130354
	Reverse	GCAGAGCCTCCACCTTGTAG			

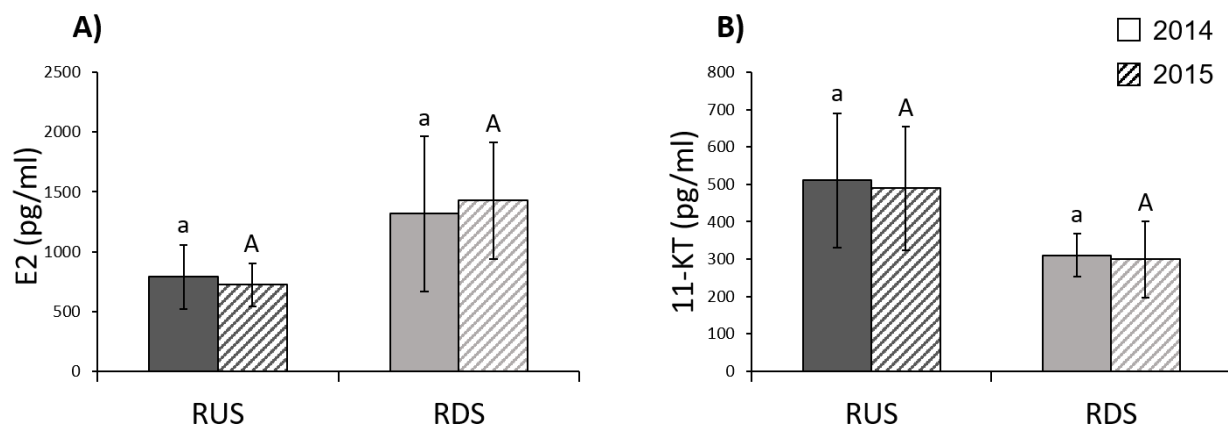
### **3.3.4 Statistical Analysis**

All analysis was conducted using SPSS Version 20 (IBM Corp., Armonk, NY, USA). Male and female fish were analyzed separately. Normality of the data was tested using the Shapiro-Wilk test and for homogeneity of variance was tested using the Levene's test. Data that did not meet the assumptions of a parametric test were logarithmically transformed and re-analyzed using parametric statistics. When data could not be normalized, the Kruskal-Wallis test followed by the Mann Whitney U post hoc test were applied. Normally distributed data was analyzed using a one-way analysis of variance (ANOVA) followed by Tukey's post hoc test to determine if there was significant variation among and within the sampled sites for all parameters investigated. A probability of  $p \leq 0.05$  was used to determine statistical significance.

## **3.4 Results**

### **3.4.1 Plasma hormone concentrations**

E2 concentrations for females were not significantly different between RUS and RDS for either year (2014:  $p=0.475$ ; 2015:  $p=0.392$ ) despite a trend of increasing concentrations in fish downstream of the effluent fallout (Figure 3.2). The greatest mean E2 concentration was 1427 pg/mL measured at RDS in 2015, and the lowest mean concentration of 721 pg/mL was measured at RUS in 2015. There was a decreasing trend of 11-KT concentrations in males at RDS in both 2014 and 2015; however, no significant differences were found (2014:  $p=0.190$ ; 2015:  $p=0.173$ ). Males in 2014 at RUS had the greatest mean 11-KT concentration of 511 pg/mL, while the lowest mean of 300 pg/mL was measured at RDS in 2015. Due to samples needing to be pooled for analysis, variability increased, possibly explaining this trend without significance.



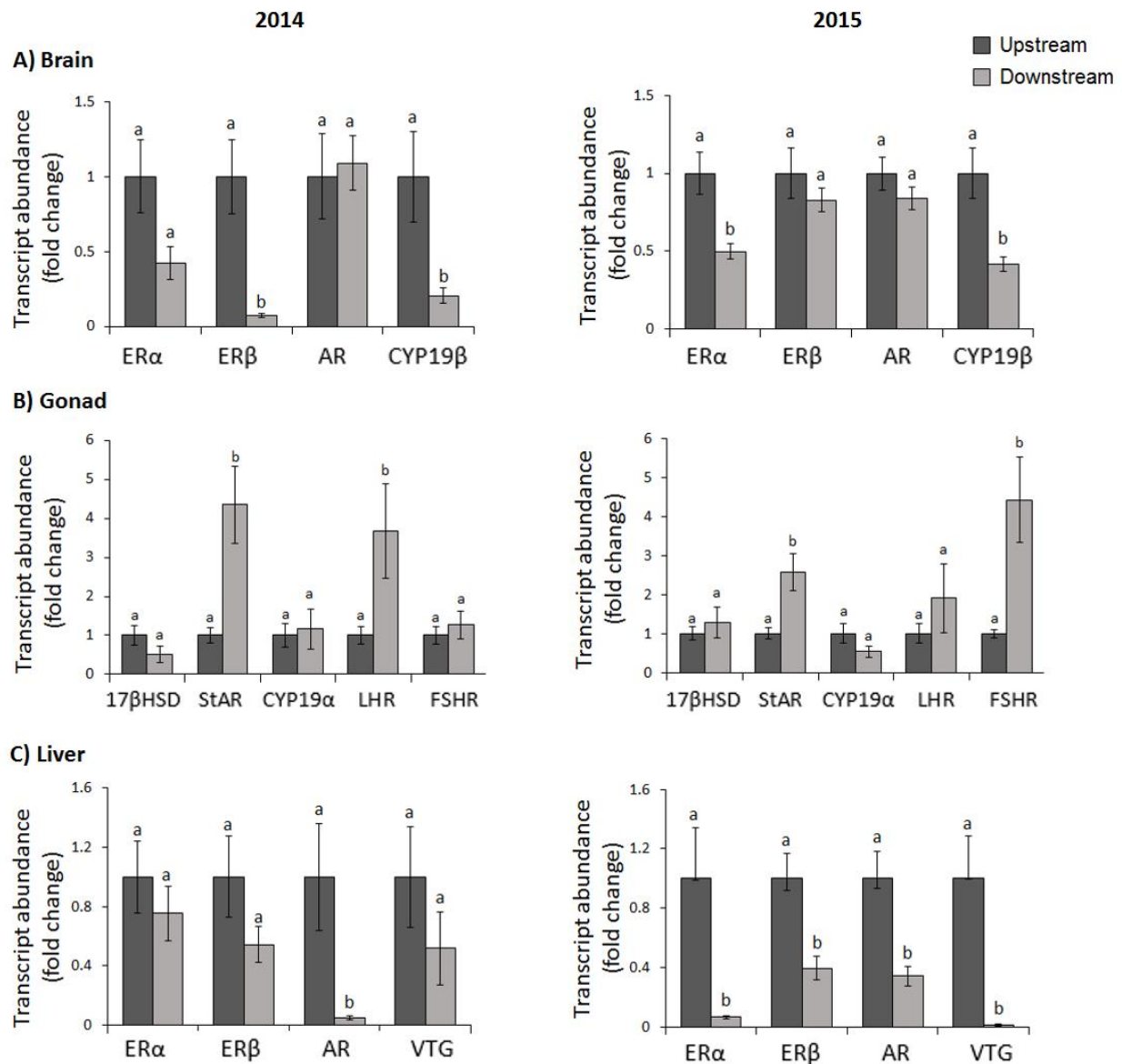
**Figure 3.2.** Mean ( $\pm$  SEM) concentrations of plasma hormones (A) 17 $\beta$ -estradiol (E2) in female, and (B) 11-ketotestosterone (11-KT) in male fathead minnows collected upstream (RUS) and downstream (RDS) of the effluent fallout in Wascana Creek, SK in 2014 (solid bars) and 2015 (dashed bars). Different letters indicate significant difference in the concentrations of plasma hormone between sites ( $p \leq 0.05$ ).

### 3.4.2 Effects on gene expression in female fish

Exposure to MWWs at RDS significantly affected the abundances of transcripts of target genes expressed in all tissues of female fathead minnows. In brains, the abundances of ER $\alpha$ , ER $\beta$  and CYP19b at RDS were significantly less by  $0.42 \pm 0.12$ ,  $0.07 \pm 0.01$ , and  $0.20 \pm 0.05$  fold, respectively, than in brains from females at RUS in 2014 (Figure 3.3A). Similarly, in 2015, abundances of ER $\alpha$  and CYP19b were significantly lower by  $0.50 \pm 0.05$  and  $0.42 \pm 0.05$ , respectively, in females at RDS. No effects were seen on expression of AR downstream in either 2014 or 2015.

Transcript abundances were also affected in the gonads of females at RDS compared to RUS (Figure 3.3B). In both 2014 and 2015, the abundance of StAR was significantly greater by  $4.34 \pm 0.98$  and  $2.58 \pm 0.48$  fold, respectively, in females downstream compared to upstream. In 2014, the abundance of LHR was greater by  $3.67 \pm 1.21$  fold in females at RDS. However, in 2015, no change was observed in LHR, but rather a significant increase in the transcript FSHR by  $4.43 \pm 1.10$  fold was seen downstream. Abundances of transcripts 17 $\beta$ HSD and CYP19a were not different in the gonads of female fish at RDS.

Exposure to MWWs at RDS resulted in a statistically significant reduction of transcript abundance of ER $\alpha$  and ER $\beta$  in liver by  $0.06 \pm 0.01$  and  $0.40 \pm 0.08$  fold, respectively, in females collected during 2015, while no change was observed in estrogen receptors in female livers collected during 2014 (Figure 3.3C). Similar to the other tissues, the transcript AR did not differ in abundance in liver in 2014; however, abundance of AR was  $0.34 \pm 0.06$  fold less in 2015 in females at RDS. In 2014, VTG abundance was not different between sites. Conversely, abundance of VTG in 2015 was significantly reduced by  $0.01 \pm 0.01$  fold in livers of females at RDS.



**Figure 3.3.** Abundances of transcripts of genes involved in sex steroid hormone synthesis and signaling in female fathead minnows upstream (RUS; dark) and downstream (RDS; light) of the effluent fallout in Wascana Creek, SK in 2014 (left; RUS:  $n = 17$ , RDS:  $n = 10$ ) and 2015 (right; RUS:  $n = 16$ , RDS:  $n = 26$ ). (A) brain, (B), gonad, (C) liver. Data are reported as the mean  $\pm$  SEM. Statistical significance denoted by \* =  $p \leq 0.05$ .

### 3.4.3 Effects on gene expression in male fish

Male fathead minnows collected at RDS exposed to MWWs also displayed differences in the abundances of transcripts of target genes expressed in all tissues. In the brain, significant changes in the abundances of transcripts of target genes were observed (Figure 3.4A). The abundances of ER $\beta$  and CYP19b were down-regulated by  $0.17 \pm 0.03$  and  $0.10 \pm 0.02$  fold, respectively, in males at RDS compared to males collected at RUS in 2014. In 2015, expression of both estrogen receptors, ER $\alpha$  and ER $\beta$ , were  $0.35 \pm 0.06$  and  $0.45 \pm 0.06$  fold less, respectively, in downstream fish compared to RUS. No changes in the abundance of AR were observed in either year.

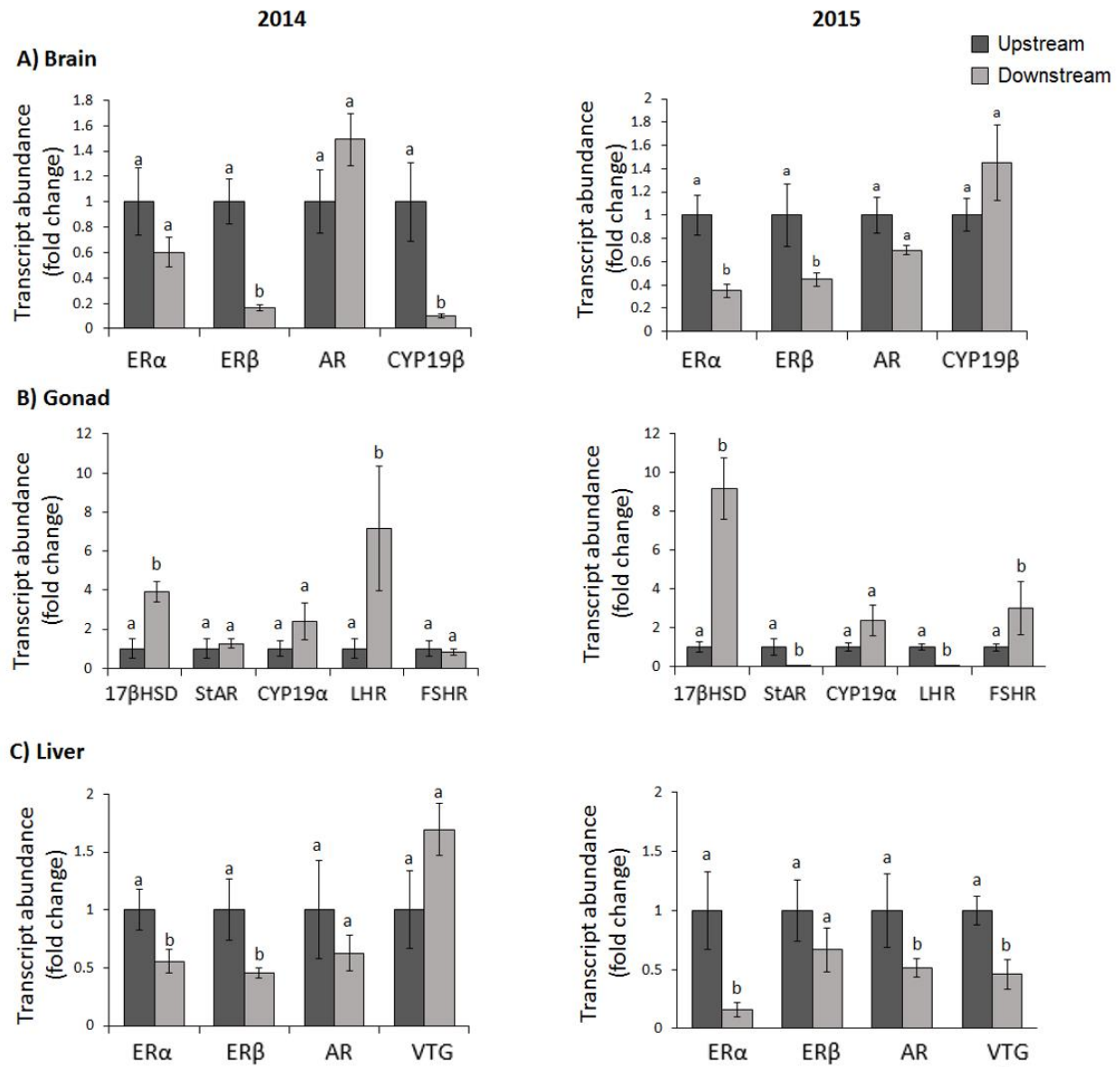
Exposure to effluents downstream at RDS affected abundances of transcripts of a number of target genes expressed in gonads from male fathead minnows when compared to fish collected upstream (Figure 3.4B). In both 2014 and 2015, abundance of the transcript 17 $\beta$ HSD was significantly increased by  $3.89 \pm 0.51$  and  $9.18 \pm 1.58$  fold, respectively, at RDS. Additionally, for both years, no changes occurred in transcript abundance of CYP19a. Differences among years were seen in the abundance of StAR, LHR and FSHR. For 2014, a significant induction of LHR abundance was observed; however, abundances were not different for StAR or FSHR at RDS. Alternatively, in 2015, abundances of StAR and LHR were significantly less by 0.01 and 0.0002 fold, respectively, but abundance of FSHR was significantly greater by 4.43 fold in male gonad tissue at RDS compared to RUS.

Abundances of target genes in liver tissue were also affected in males at RDS compared to RUS (Figure 3.4C). For the 2014 sampling, abundances of transcripts ER $\alpha$  and ER $\beta$  were significantly less by  $0.55 \pm 0.10$  and  $0.45 \pm 0.05$  fold, respectively, and no change was seen in AR. Significantly lowered abundances of target genes in the liver were also found in 2015, with greatly

reduced transcript levels of ER $\alpha$  and AR, but no differences measured in ER $\beta$  in males at RDS.

The expression of VTG in male livers was not different in 2014, however in 2015, a significant reduction of  $0.46 \pm 0.13$  fold was found at RDS.





**Figure 3.4.** Abundances of transcripts of genes involved in sex steroid hormone synthesis and signaling in male fathead minnows upstream (RUS; dark) and downstream (RDS; light) of the effluent fallout in Wascana Creek, SK in 2014 (left; RUS: n = 10, RDS: n = 24) and 2015 (right; RUS: n = 6, RDS: n = 4). (A) brain, (B), gonad, (C) liver. Data are reported as the mean  $\pm$  SEM. Statistical significance denoted by \* =  $p \leq 0.05$ .

### 3.5 Discussion

This field study identified significant molecular alterations by which contaminants in MWWs in Wascana Creek may have elicited the apical outcomes reported in parallel study (Chapter 2) in resident fathead minnow populations. While minimal differences were observed in plasma hormones, very similar trends were detected between years and sampling sites, and significant changes were measured in the expression of key genes along the entire HPGL axis. These molecular findings support the apical outcomes from Hanson et al., (Chapter 2), which showed adverse biological effects such as altered condition, increased liver sizes, delays in maturation, and degradation of reproductive tissues in FHM's downstream of the City of Regina's WWTP.

Quantifying plasma sex steroid hormone concentrations is well utilized due to its ease of measurement and defined role of these hormones in gametogenesis and spawning (Folmar et al., 1996). Fathead minnows are asynchronous spawners, with spawning events occurring June through August, depending on climatic and flow conditions, in Wascana Creek. Unlike synchronous spawners, such as rainbow trout, the spermatogenic and oogenic activity in the testes and ovaries, respectively, of fathead minnows remains relatively constant throughout the spawning season (Kobayashi and Aida, 1986; Nichols et al., 1999). In our study, while not significant, slight increases in E2 concentrations were consistently observed in females downstream of the effluent fallout compared to upstream. While increases in circulating sex steroid hormones are associated with initiation and maintenance of gonad maturation (Lee and Yang, 2002; Schulz et al., 2010), increases have also been observed with exposure to MWWs (Folmar et al., 2000; Tilton, et al.,

2002; Tetreault, G., 2012). The opposite trend was observed for males at RDS, with decreasing trends in 11-KT concentrations compared to RUS during both sampling years. 11-KT is crucial at regulating reproductive physiology in male fish and correlations have been made between increasing 11-KT levels and maturation state in male teleosts (Beitel et al., 2015; Fitzpatrick et al., 1986). In male fish exposed to an anti-androgen mixture comprising of a common pesticide, linuron, and plasticizer, DEHP, found in MWWs, lowered testosterone (T) plasma levels were observed, hypothesized to be due to increased steroid catabolism (Crago and Klaper, 2012). Additionally, gemfibrozil, a pharmaceutical commonly found in MWWs and measured at high concentrations in Wascana Creek (Chapter 2; Tetreault et al., 2011), has been shown to reduce testosterone levels in fish by disrupting StAR expression in the gonads (Mimeault et al., 2005). Lowered expression of StAR in the gonads, potentially due to exposure to PPCPs such as gemfibrozil, was also observed in this study in 2015 males and may have contributed to the reductions in 11-KT concentrations. However, due to plasma samples needing to be pooled for analysis, variability increased and our sample size was too low to draw firm conclusions. Therefore, further studies with greater sample numbers are essential to investigate the potential of the City of Regina MWW on this endpoint to ensure that these differences are not only reflective of the regular and transient changes occurring in diel hormone concentrations. While we were able to describe the maturation status of fish at each site through histological staging (Chapter 2), it would be beneficial to characterize a full seasonal profile of plasma hormones to further support our findings.

The receptors to which sex steroid hormones bind are often the target of EDCs, resulting in agonistic effects on cellular processes or alternatively antagonistically binding to and blocking the receptor binding sites, therefore blocking the transcriptional activation triggered through these

receptors (Arukwe, 2001). Increases in mRNA levels of estrogen receptor genes (ER $\alpha$  and ER $\beta$ ) have been shown in response to exposure to estradiol, the synthetic estrogen, 17 $\alpha$ -ethinylestradiol (EE2), and the anti-androgen, flutamide (Bowman et al., 2000; Filby et al., 2007). However, in this study, FHM populations in both 2014 and 2015 at RDS had decreased expression of both estrogen receptors measured in brain and liver tissue, suggesting that the changes observed may not be due to the direct action of estrogenic or anti-androgenic compounds. In addition to changes observed on the ER subunits, Filby et al. (2007) observed decreases in expression of the androgen receptor in both the liver and gonad tissues with exposure to the anti-androgen, flutamide. Similarly, in this study, we also found down-regulation of AR in the livers of both females and males. Additionally, expression of VTG, an estrogen responsive gene in the liver, shown to increase with estrogen exposure, was significantly downregulated in females at RDS in 2015, with a similar but non-significant trend in 2014, suggesting further that exposure to MWWs in Wascana Creek was not specifically estrogenic. Decreases in VTG in female fathead minnows have been shown with exposure to androgen agonists (17 $\alpha$ -trenbolone and 17 $\beta$ -trenbolone), an estrogen antagonist (fenarimol) and inhibitors of steroidogenesis including fungicides (prochloraz and fenarimol) and pharmaceuticals (fadrozole; Miller et al., 2007). Furthermore, VTG induction in males is a highly sensitive biomarker of estrogenic exposure in fish and used extensively in both laboratory and field settings (Vajda et al., 2008; Folmar et al., 1996; Palace et al., 2002); however, in this present study, VTG expression was not increased in males at RDS further suggesting that estrogenic potentials were not present at relevant levels in Regina MWW. This lack of estrogenic effects is further supported with estrogenic compounds being absent in effluent chemical analysis (Chapter 2) and lack of estrogenic activity in *in vitro* bioassays conducted by Bagatim (2019) with City of Regina MWWs.

In addition to measurements of expression of hormone receptors in brain and liver tissues, abundance of genes of two membrane-bound gonadotropin receptors, luteinizing hormone receptor (LHR) and follicle-stimulating hormone receptor (FSHR), were measured in gonadal tissues. LHR and FSHR expression was significantly increased in 2014 and 2015, respectively, for both males and females. Increases in LHR and FSHR expression may be in response to increased release of FSH $\beta$  and LH $\beta$  from the pituitary in response to exposure to the MWWs. Many studies have shown FSHR- and LHR-activating properties by FSH and LH, respectively (Han et al., 1996; He et al., 2012; Grossman et al., 1997). In contrast, lowered LHR expression in males at RDS in 2015 might be a feedback mechanism of regulating steroidogenesis in response to a greater concentration of circulating LH.

In addition to effects observed on specific receptors, evidence of inhibition of steroidogenesis was also present in this study when assessing the expression of enzymes. In this study, aromatase, a cytochrome P450 enzyme responsible for the production of E2 by catalyzing its conversion from T, was suppressed in brain tissue (CYP19 $\beta$ ), but unchanged in the gonads (CYP19 $\alpha$ ). Differences in expression of CYP19 genes between tissues has been previously reported (Govoroun et al., 2001b; Cheshenko et al., 2008). Increases in CYP19 $\beta$  have been widely reported with exposure to estrogens, and to a lesser extent, anti-androgens (reviewed in Callard et al., 2001; Filby et al., 2007). Aromatase inhibitors include a variety of chemicals found in the environment such as pesticides, organochlorines and organotins, some of which have been shown to lead to prevalence of male phenotypes in exposed populations and inhibit ovarian and testicular growth in fathead minnows (Ankley et al., 2002; Villeneuve et al., 2006; Cheshenko et al., 2008). Decreased expression of aromatase in the brain found in this study paired with lowered GSI and inhibited maturation of gonads (Chapter 2) found in populations at RDS suggests inhibition of

steroidogenesis potentially due in part to aromatase inhibitors found in the MWWEs. In this study, expression of 17 $\beta$ -hydroxysteroid dehydrogenase (17 $\beta$ -HSD), an enzyme involved in regulating the levels of active androgens and estrogens, was found to be up-regulated (9 fold) in males, but not females, collected from RDS. Similar findings of increased expression of 17 $\beta$ -HSD are not prevalent in the literature; however, inhibitory effects on 17 $\beta$ -HSD have been observed with exposure to EE2 and are consistent with the mechanism of action of estrogens inhibiting T and 11-KT production (Filby et al., 2007). As shown in this study, male FHMs from RDS showed consistently decreasing trends in plasma 11-KT concentrations; therefore, it is possible the organisms tried to upregulate 17 $\beta$ -HSD as a compensatory act to increase hormone production. In steroidogenesis, the rate-limiting step of supplying cholesterol, the precursor for steroids, to the inner mitochondrial membrane is mediated by steroidogenic acute regulatory protein (StAR). Expression of the gene encoding for this protein was increased in females during both sampling years, but decreased in males, which is thought to have led to the decreasing trend in plasma 11-KT as described above. Differences between sexes were unexpected as StAR mediates processes that are common to the synthesis of estrogens, androgens and progestins; however, this may suggest that these changes were of a secondary nature and not directly affected by the chemicals present in Wascana Creek downstream of the WWTP of Regina. Similar to our study, Ings et al. (2011) also found increased expression of StAR in fish downstream of a WWTP hypothesized to be a stress response, increasing its capacity to produce cortisol. Furthermore, the increased expression of StAR in females may also be viewed as part of a compensatory response to the effects of the MWWEs, by which increasing the transfer rate of cholesterol from outer to inner mitochondrial membrane if translation of the protein was carried through. While increases in StAR mRNA levels have been shown with exposure to xenoestrogens (Arukwe, 2008; Vang et al., 2007),

it is likely that in this study, this increase is rather part of a stress or compensatory response from exposure to multiple stressors in the effluents.

Although endogenous and synthetic hormones were not detected in surface waters downstream of the effluent fallout (Chapter 2), a number of other compounds with endocrine potential activity were found at elevated concentrations. The four most predominant compounds included carbamazepine, DEET, gemfibrozil and triclosan.

Carbamazepine and gemfibrozil, both anti-androgenic compounds, have been shown to lower androgen levels in fish with chronic exposure, but at levels much higher than detected at RDS, suggesting that the molecular effects observed are likely not due to the individual concentrations (Fraz et al., 2018; Mimeault et al., 2005). While reduced expression of the androgen receptor has been observed in female FHMs following exposure to DEET at 600 ng/L (Zenobio et al., 2014), concentrations of DEET at RDS were lower (172.2 ng/L; Chapter 2). Thus, it is unlikely that the concentrations of DEET measured alone would account completely for the reduction in AR expression in liver tissues of RDS females in both 2014 and 2015. Triclosan, a compound that is structurally similar to estrogenic and androgenic EDCs (Veldhoen et al., 2006), has been shown to disrupt the endocrine system of fish at concentrations similar to what was observed in this study (Chapter 2; Raut and Angus 2010). Significantly decreased expression of both ER and AR in male carp has been shown with exposure to triclosan (Wang et al., 2017), and may explain the suppressed expression of these receptors in males at RDS for both 2014 and 2015. While most of the chemicals detected downstream of the City of Regina's WWTP were detected at concentrations previously reported as "non-toxic", these chemicals co-occur with each other as well as a myriad of other compounds in a complex mixture with little known regarding the potential effects when all combined (Chapter 2).

Overall, the present study trends of general suppressive responses are consistent with apical findings in a parallel study (Chapter 2) such as delays in maturation, degeneration of reproductive tissues and altered condition of FHM's downstream of the City of Regina's WWTP.

### **3.6 Conclusions**

Exposure of resident female and male fathead minnows to MWWs downstream of the City of Regina's WWTP resulted in non-significant trends in changes in plasma hormone concentrations; however, significant changes were present in abundances of transcripts at every level of the HPGL axis. This is not entirely surprising as the make-up of MWWs is quite complex containing a multitude of compounds with endocrine disrupting capabilities. Due to decreased expression of estrogen responsive genes, ER $\alpha$ , ER $\beta$  and VTG, estrogenic compounds likely did not cause the effects observed. This is consistent with findings found in three parallel studies that demonstrated diverse biological effects such as altered condition, increased liver sizes, delays in maturation and degradation of reproductive tissues, but no estrogenic effects or potentials (Chapter 2; Bagatim, 2019; Steeves, 2018). Alternatively, down-regulation of key steroidogenic genes, CYP19 $\beta$  and StAR, coupled with lowered FSHR and LHR expression, suggested that steroidogenic capacity in the gonads of fish exposed at RDS had been reduced.

Overall, this study's results of observed trends of general suppressive molecular responses provide mechanistic evidence for the apical findings in a parallel study (Chapter 2) such as delays in maturation, histological alterations and reduced secondary sexual characteristics of fathead minnows exposed to MWWs in Wascana Creek. The general inhibition of reproductive functions of FHMs at RDS is in accordance with two parallel studies with lab exposed fish showing reduced



fecundity in addition to similar delays in maturation and predominant antagonistic potentials detected by the ER and AR receptor assays (Bagatim, 2019; Steeves, 2018). Taking in all the findings of this study as well as the parallel, and the overall lack of estrogenic effects, it is likely that the effects observed were not due to estrogenicity in the effluents. This was further supported by the low estrogenic potential of the effluents entering RDS reported by the *in vitro* assays (Bagatim, 2019). Key biomarkers of estrogenic exposure, such as induction of VTG and intersex were absent as well as lack of detection of estrogenic compounds in surface water. With the complex nature of MWWs, it is likely the suppressive responses observed in this study are due to stress from the varying compounds associated with these effluents. It should be noted, that it cannot be discounted that one or more of the compounds present in the effluents could be acting at the protein level, and therefore future studies should evaluate this further.

## Chapter 4 : GENERAL DISCUSSION

### 4.1 Introduction

In recent decades there has been a significant increase in concern surrounding the introduction of numerous compounds into aquatic environments, and their potential to alter the endocrine system of fish and other aquatic species that inhabit them. The release of MWWs into these systems is of particular concern as they contain complex mixtures of contaminants, many of which are considered to be EDCs and have been shown to affect growth, development and reproduction of aquatic organisms, particularly fish, through disruption of endocrine homeostasis, sometimes at very low concentrations (Campbell et al. 2006; Hecker et al., 2006; Goksøyr 2006). In semi-arid regions, such as the Canadian prairies, increased risk may be present as receiving waterbodies of these compounds are often characterized by low flow and minimal dilution (Cwiertny et al., 2014; Dotan et al., 2016; Waiser et al., 2011). Wascana Creek, SK, Canada was chosen as a representative system for small semi-arid prairie environments as it is an effluent dominated stream that receives MWWs from the City of Regina's outdated lagoon-based treatment facility and has been shown to contain up to 100% treated effluent during low flow periods.

The purpose of the research conducted in this thesis was to evaluate the potential impacts of MWWs on reproductive health and endocrine homeostasis of wild fathead minnow (*Pimephales promelas*) populations downstream of the City of Regina's WWTP in Wascana Creek, Saskatchewan, Canada. In particular, the first objective was to compare the maturation status, reproductive health and endocrine status between populations up- and down-stream of the City of Regina. The second objective was to compare between sampling years; 2014 and 2015.

The final objective was to determine if effects were observed in FHM populations downstream, if they could be correlated to exposure to certain chemicals present in MWWs released from the City of Regina's WWTP during the time of sampling. Furthermore, this study was part of a large scale research effort known as the Aquatic Impact Assessment of Municipal Effluents (AIME). The AIME project contained multiple tiers, including (1) *in vitro* bioassays to identify endocrine activity of effluents (2) a short-term fish reproductive bioassay with effluent exposures in laboratory and (3) wild-fish surveys in an effluent-dominated system. The overall goal of this research initiative was to establish effective endpoints and develop a set of techniques or a "toolbox" for assessing the potential impacts of MWWs on aquatic organisms.

## **4.2 Summary of Findings**

This present study used multiple lines of evidence to evaluate the potential impacts of MWWs on the reproductive health and endocrine homeostasis of wild fathead minnow populations downstream of the City of Regina's WWTP in Wascana Creek, Saskatchewan, Canada. As discussed within the previous chapters of this thesis, endpoints were evaluated from molecular level to whole organism level described below and summarized in Table 4.1.

The overall health (condition factor, ovosomatic indices) and reproductive (secondary sexual characteristics, gonad histopathology) statuses were assessed for spawning fathead minnow populations downstream of the effluent fallout in Wascana Creek, SK, Canada (2014 and 2015). It was observed that their overall reproductive fitness was likely impacted with exposure to MWWs from the City of Regina. This was evidenced by FHMs downstream of the effluent having lower gonadosomatic indices (GSI) and significantly greater hepatosomatic (HSIs)

compared to upstream populations and lowered scores of secondary sexual characteristics. It is likely the reductions observed in GSIs observed in males and females downstream are due to a number of different stressors, such as high nutrient levels, a number of different EDCs and most importantly, the high concentrations of ammonia (0.241 and 0.102 mg/L) in the effluents released into the system during the sampling period. Changes in HSIs, an indication of energy status, observed in this study are likely attributed to the hypothesis of induction of liver detoxification enzymes with exposure to toxic contaminants associated with the MWWs, and not due to vitellogenin induction as some studies have reported, because of the consistent absence of estrogenic effects in our endpoints as well as the lack of detection of estrogenic compounds in surface water. Additionally, these increases may be explained by the elevated concentrations of both nitrogen and phosphorus downstream as observed in Waiser et al., (2011). The lowered secondary sexual characteristics in male FHMs collected downstream compared to upstream suggest demasculinization of males as development of these are androgen dependent. Disruption in this normal development may negatively impact key spawning behaviours and adversely impact the fitness of the fish in territorial contests, access to mates or quality of parent care (Grist et al., 2003; Lange et al., 2011 and Scott and Sloman, 2004). Steeves (2018) found no changes in morphometrics measured in the parallel study with lab exposed fish, however reduced fecundity was observed.

Additionally, in both females and males there was significantly greater occurrence and severity of gonadal degradation and delayed maturation in downstream fish compared to upstream fish; however, no indications of exposure to estrogenic compounds such as occurrence of testicular oocytes were observed. Delays in gonadal maturation were well documented in both females and males at RDS with similar alterations being observed by Steeves (2018) in lab exposed organisms.

Similar delays have been well document in wild fish exposed to treated sewage effluent (Diniz et al., 2005; Douxfils et al., 2007). The changes in the gonadal tissue structure or development may have long-term implications for reproductive output in affected organisms. Interestingly, the well documented phenomenon of mixed sex or intersex in fish exposed to MWW (darters: Tetreault et al., 2011; roach: Bjerregaard et al., 2006; mirror carp: Diniz et al., 2005; roach: Jobling et al., 1998; medaka: Zha et al., 2006), was not evident in males collected downstream, suggesting that populations were not exposed to estrogenic compounds at concentrations great enough to cause this condition. This is further supported with the lack of detection of reproductive hormones or estrogenic agonists in the surface water samples collected at RDS.

The underlying molecular alterations that may have elicited the higher level biological effects were investigated in Chapter 3. While histopathological changes were observed in the gonads for both sexes downstream, there were only minimal changes observed in plasma hormone levels. This lack of response was also observed by Steeves (2018) suggesting that the observed apical outcomes in these *in vitro* exposure studies (i.e. histopathology, fecundity, morphometrics) were not driven by changes in plasma hormone levels. However, the increased variability due to pooling of samples makes it difficult to draw firm conclusions.

While significant alterations in the expression of multiple target genes along the HPGL axis were observed in FHM populations downstream for both 2014 and 2015, there was not a clear mechanism by which the MWWs caused the apical effects observed. Most notably, the expression of vitellogenin (VTG), a highly sensitive biomarker of estrogenic exposure in fish, was not elevated in males downstream in either the wild or lab exposed fish (Steeves, 2018). While inductions of VTG are widely shown in studies with fish exposed to MWW or wild fish collected downstream WWTP outfalls (Barber et al., 2007; Harries et al., 1999), decreases in VTG have also

been reported with exposure to androgen agonists, estrogen antagonists and inhibitors of steroidogenesis (Miller et al., 2007). The latter of which may explain the significant downregulation of VTG in females downstream in 2015. Interestingly, decreased expression of both estrogen receptors were measured in brain and liver tissues. Increases in these genes have been observed with exposure to estrogens as well as anti-androgens (Bowman et al., 2000; Filby et al., 2007), suggesting that the changes observed at RDS may not be due to the direct action of estrogenic or anti-androgenic compounds. Furthermore, in this study we found down-regulation of AR in the livers of both females and males. This lowered expression has been shown in both liver and gonad tissues with exposure to the anti-androgen, flutamide (Filby et al., 2007). The inhibitory action in the expression of the AR and ER suggest that antagonistic compounds may be the primary drivers of endocrine potentials in Regina MWWs. These findings are supported by results of ER and AR receptor assays showing predominant antagonistic potentials of the effluents (Bagatim, 2019).

In addition to effects observed on specific receptors, evidence of inhibition of steroidogenesis was also seen. Aromatase, an enzyme responsible for the production of E2 was suppressed in brain tissue (CYP19 $\beta$ ), but unchanged in gonads (CYP19 $\alpha$ ). This decrease in expression, coupled with lowered GSI and delayed maturation of gonads found in RDS populations, suggests inhibition of steroidogenesis is potentially due in part to aromatase inhibitors found in the MWWs. Expression of 17 $\beta$ -hydroxysteroid dehydrogenase (17 $\beta$ -HSD), an enzyme involved in regulating the levels of active androgens and estrogens, has been shown to decrease with exposure to EE2, however in this study, males downstream had increased expression of 17 $\beta$ -HSD suggesting a compensatory act was being introduced to increase hormone production. In the process of steroidogenesis, the rate limiting step is mediated by steroidogenic acute regulatory

protein (StAR) which has been shown to increase with exposure to xenoestrogens as well as hypothesized to be a stress response, increasing the organisms capacity to produce cortisol (Arukwe, 2008; Ings et al., 2011; Vang et al., 2007). It is likely in this study that the latter is responsible for the increases observed in StAR expression in females downstream of the effluent fallout with multiple stressors in the effluents and not due to estrogenic compounds specifically.

Overall, this research study observed trends of general suppressive molecular responses that are consistent with the apical findings such as delays in maturation, degeneration of reproductive tissues and altered conditions of FHM's downstream of the City of Regina's WWTP.

**Table 4.1.** Summary of responses for whole organism, tissue, steroid hormones and gene expression for female (F) and male (M) fathead minnows collected downstream of the effluent fallout in Wascana Creek, SK (RDS). (↑) statistically significant increase, (↓) statistically significant decrease, (-) difference from control group is not statistically detectable.

		2014		2015		Recommend as Health Screening Biomarker
		F	M	F	M	
Whole Organism						
	Condition Factor	-	↑	↓	↑	NO
	GSI	↓	↓	↓	-	YES
	HSI	↑	↑	↑	↑	YES
	2 <sup>o</sup> Sex Characteristics	N/A	↓	N/A	↓	YES
Tissue						
	Spermatogonia	N/A	↑	N/A	N/A	YES
	Testicular Degeneration	N/A	↑	N/A	N/A	YES
	Gonadal Staging	↓	N/A	↓	N/A	YES
	Oocyte Atresia	↑	N/A	↑	N/A	YES
Steroid Hormones						
	E2	-	N/A	-	N/A	NO
	11-KT	N/A	-	N/A	-	NO
Gene Expression						
Brain	ERα	-	-	↓	↓	YES
	ERβ	↓	↓	-	↓	YES
	AR	-	-	-	-	YES
	CYP19β	↓	↓	↓	-	YES
Gonad	17βHSD	-	↑	-	↑	YES
	StAR	↑	-	↑	↓	NO
	CYP19α	-	-	-	-	YES
	LHR	↑	↑	-	↓	NO
	FSHR	-	-	↑	↑	NO
Liver	ERα	-	↓	↓	↓	YES
	ERβ	-	↓	↓	-	YES
	AR	↓	-	↓	↓	YES
	VTG	-	-	↓	↓	YES



### 4.3 AIME Project Findings

The findings from this thesis research were used as part of the multi-tiered research project, the Aquatic Impact Assessment of Municipal Effluents (AIME). In addition to this thesis research, two additional parallel projects were conducted that also examined potential impacts of the City of Regina's effluents by (1) using *in vitro* bioassays to characterize the specific endocrine activity of the effluents (Bagatim, 2019) and (2) conducting short-term fish reproductive bioassays in the laboratory with collected effluents in 2014 (Steeves, 2018). While not all impacts of effluent exposure observed and any associated mechanisms of action in fish are consistent across the three parallel studies, there are a number of uniformities across endpoints in each tier that are discussed below. The effects of the MWWs from the City of Regina's WWTP in all three parallel studies are summarized in Figure 4.2.

Similar histological observations of increased spermatogonia in males and increased oocyte atresia in females, indicating tissue-level changes in the gonads, were observed in fish collected downstream of the Regina WWTP (this thesis research) as well as in fish exposed to Regina effluents in the laboratory (Steeves 2018). Similarly, both studies found no changes in plasma sex steroid hormone concentrations as well as no induction of VTG when exposed either *in situ* or in lab-exposure to Regina effluents. Taking into account all findings in this study, and lack of estrogenic effects, it is hypothesized that the effects observed in this present study were likely not due to estrogenic compounds in the effluent. These conclusions are consistent with those found in the parallel studies by Steeves (2018) and further supported by Bagatim's (2019) findings of low estrogenic specific potentials of effluents reported by the *in vitro* assays. Additionally, there was a lack of agonistic potentials in the ER transactivation assay (Bagatim, 2019) and no detection of female reproductive hormones or other chemicals with the ability to act

as estrogens in the surface waters downstream (Chapter 2) that have previously been identified as the main causative agents for estrogenic activities in MWWs.

While there was no evidence of androgenicity occurring, such as masculinization of female fish through the development of nuptial tubercles, there was evidence of anti-androgenicity in both the wild-caught and lab-exposed fish. Delays in gonadal maturation were observed in both studies, and decreased nuptial tubercle scores and lowered androgen receptor expression were seen in wild exposed fish. The anti-androgenic potential of Regina effluents are further supported by the third study where Bagatim (2019) observed significant anti-androgenic potential in Regina effluents *in vitro*.

It is well documented in the literature that MWWs are highly complex and variable mixtures, and as evidenced in the three parallel studies it is invaluable to assess them using multiple endpoints such as those highlighted in the tiered AIME project to properly assess reproductive effects on aquatic organisms.

**Table 4.2.** Summary of responses for entire AIME project with in vivo (wild fish studies, this thesis; 21 reproductive assay, Steeves, 2018) endpoints for female (F) and male (M) fathead minnows and in vitro bioassay potentials (Bagatim, 2019). (↑) statistically significant increase, (↓) statistically significant decrease, and (--) not applicable.

Endpoints	City of Regina Effluent Effect?			
<i>In Vivo</i>	Wild Fish		Reproductive Assay	
	F	M	F	M
Condition (K; Effect Size 10%)	No	No	No	No
Relative Liver Size (HSI; Effect Size 25%)	Yes ↑	Yes ↑	No	No
Relative Gonad Size (GSI; Effect Size 25%)	Yes ↓	Yes ↓	No	No
Fecundity (females)	--	--	Yes	--
Fertility	--	--	No	No
Intersex Severity	No	No	No	No
Histopathology (maturation and degradation)	Yes	Yes	Yes	Yes
Circulating Hormones	No	No	No	No
VTG Gene Expression	No	No	No	No
ER Gene Expression	Yes ↓	Yes ↓	Yes ↑ <sup>b</sup>	No
AR Gene Expression	Yes ↓ <sup>a</sup>	Yes ↓ <sup>a</sup>	Yes ↑ <sup>b</sup>	No
Aromatase Expression	Yes ↓ <sup>b</sup>	Yes ↓ <sup>b</sup>	Yes ↑ <sup>a</sup>	No
Stable Isotopes				
Nitrogen	Yes ↓	Yes ↓	--	--
Carbon	Yes ↑	Yes ↑	--	--
<i>In Vitro</i>	Bioassay			
Effluent Androgenicity	No			
Effluent Estrogenicity	Yes (low)			
Effluent Anti-Androgenicity	Yes			
Effluent Anti-Estrogenicity	Yes			
Effluent Steroidogenesis Disruption	Yes			

<sup>a</sup> Liver tissue only

<sup>b</sup> Brain tissue only

## 4.4 Future Work

While this thesis research featured a comprehensive study of the impacts of exposure to MWWs in wild fish populations from the molecular to the organismal level, there are a number of areas within the studies conducted that could be expanded to provide further insight into mechanisms and sources of the effects observed.

When evaluating the increases in liver sizes (Chapter 2), it is possible that changes observed may not only be influenced by the elevated nutrient concentrations, but also changes in lipid or glycogen storage and food supply. To further evaluate this, investigation into liver enzymatic activity and energy stores would be beneficial in determining this relationship. Additionally, as the liver is one of the major detoxification organs and is involved in metabolism of xenobiotics, the use of histopathology would be desirable to identify glycogen content, oxidative stress and indicators of stress such as melanomacrophages.

General trends without significance were observed for plasma hormones, E2 and 11-KT in this study (Chapter 3). Due to the plasma volume being limited, individual samples were pooled for plasma hormone analysis increasing the variability, potentially explaining only trends being observed. Blood was collected from the caudal vein/artery of the FHM's with a heparinized 29 gauge needle and syringe and plasma hormones measured through ELISA. The use of an alternative measurement technique, such as radioimmunoassay (RIA) could provide more robust results as it is optimized for the relatively small sample volumes obtained from FHMs; however, commercially available reagents are only available for E2 and T, not KT (Ankley et al., 2010). To increase the amount of sample and reduce the amount variability, a different approach to blood

collection could be suggested as well. One alternative method is to collect blood through caudal-cut using heparinized blood collecting tubes and then transfer to microfuge tubes.

While a thorough chemical analysis was conducted on surface water samples collected from both the upstream and downstream sites during the summer, a more complete profile over multiple seasons would provide better insights into longer term trends. Concentrations of chemicals found within the effluents have been found to vary throughout the year in WWTPs that rely on biological treatment processes, such as lagoon-based facilities like the City of Regina's. The efficiency of these processes can be reduced in certain times of the year, particularly cold winter and spring months, resulting in greater loading of contaminants and nutrients into the receiving surface water systems.

The most significant recommendation for future work would be to conduct a third additional sampling season following the completion of the upgrades at the City of Regina's WWTP. The plant at its status during the 2014 and 2015 sampling seasons was outdated and not meeting regulatory standards. However, significant upgrades were made and completed in 2017 increasing the treatment capacity and introducing a nutrient removal process. The advances in treatment would make an interesting comparison for pre- and post-upgrade MWWWE effects on resident populations in the historically effluent dominated system.

## **4.5 Conclusions**

The present study evaluated the overall health and reproductive status of resident fathead minnow populations inhabiting the effluent dominated system, Wascana Creek, SK, Canada. It was determined that wild fish downstream of the effluent fallout had adverse biological effects

such as altered condition, increased liver sizes, delays in maturation and degradation of reproductive tissues compared to populations living upstream of the City of Regina. This suggests that effluents from this WWTP pose a risk to the reproductive fitness of fish populations inhabiting the receiving surface waters. While further investigation into the mechanisms mediating these changes showed general suppression of steroidogenesis, endpoints tied definitively to estrogenic or androgenic exposure (e.g. sex steroids, VTG) were lacking. This suggests that the adverse effects observed were likely the result of multiple stressors contained in the complex effluent mixture. With continuous exposure to a diverse number of stressors including high nutrient and ammonia levels and presence of a variety of PPCPs and other contaminants, Wascana Creek should be considered as an ecosystem at high risk with regard to effects from MWWEs on resident fish populations. While this study was regional in nature, due to similarities, inferences can likely be made among different semi-arid regions around the world in regards to the ecological impacts of MWWEs.

## REFERENCES

- Ankley, G.T., Jensen, K.M., Kahl, M.D., Korte, J.J., Makynen, E.E., 2001. Description and evaluation of a short-term reproduction test with the fathead minnow (*Pimephales promelas*). *Environ. Toxicol. Chem.* 6:1276-1290.
- Ankley, G., Jensen, K., Makynen, E., Kahl, M., Korte, J., Hornung, M., Henry, T., Denny, J., Leino, R., Wilson, V., Cardon, M., Hartig, P., Gray, L. (2003) Effects of the androgenic growth promoter 17  $\beta$ -trembolone on the fecundity and reproductive endocrinology of the fathead minnow. *Environmental Toxicology and Chemistry*. 22(6): 1350-1360
- Ankley, G and Johnson, R. 2004. Small fish models for identifying and assessing the effects of endocrine disrupting chemicals. *ILAR*. 45: 469-483.
- Ankley, G., Kuehl, D., Kahl, M., Jensen, K., Linnam, A., Leino, R., Villeneuve, D. (2005) Reproductive and developmental toxicity and bioconcentration of perfluorooctanesulfonate (PFOS) in a partial-life cycle test with the fathead minnow (*Pimephales promelas*). *Environmental Toxicology and Chemistry*. 24: 2316-2324
- Ankley, G., Villeneuve, D. (2006) The fathead minnow in aquatic toxicology: past, present and future. *Aquatic Toxicology*. 78(1): 91-102
- Ankley, G., Bencic, D., Breen, M., Collette, T., Conoly, R., Denslow, N., Edwards, S., Ekman, D., Garcia-Reyero, N., Jensen, J., Lazorchak, J., Matinovic, D., Miller, D., Perkins, E., Orlando, E., Villeneuve, D., Wang, R., Watanabe, K. (2009) Endocrine disrupting chemicals in fish: Developing exposure indicators and predictive models of effects based on mechanism of action. *Aquatic Toxicology*. 92: 168-178
- Armstrong, B., Lazorchak, J., Murphy, C., Haring, H., Jensen, K., Smith, M. (2012) Determining the effects of ammonia on fathead minnow (*Pimephales promelas*) reproduction. *Science of the Total Environment*. 420:127-133
- Arukwe A. (2001). Cellular and Molecular Responses to Endocrine-Modulators and the Impact on Fish Reproduction. *Mar. Poll. Bull.* 42: 643 – 655.
- Arukwe, A., (2008). Steroidogenic acute regulatory (StAR) protein and cholesterol side-chain cleavage (P450scc)-regulated steroidogenesis as an organ-specific molecular and cellular target for endocrine disrupting chemicals in fish. *Cell Biol. Toxicol.* 24, 527-540.
- Arukwe, A and Goksoyr, A. 2003. Eggshell and egg yolk proteins in fish: hepatic proteins for the next generation: oogenetic, population, and evolutionary implications of endocrine disruption. *Comparative Hepatology*. 2: 4.

- Bagatim T., Hanson S., Steeves K., Yuan H., Wiseman S., Hogan N., Hontela A., Jones P., Giesy J. H. *In vitro* Characterization of Endocrine Disrupting Potentials of Municipal Effluents in the Prairies, Saskatchewan, Canada
- Baker, V.A. 2001. Endocrine disruptors – testing strategies to assess human hazard. *Toxicol. In Vitro*. 15, 413-419.
- Barber, L., Brown, G., Zaugg, S. 2000. In analysis of environmental endocrine disruptors. ACS Symposium Series 747. American Chemical Society: Washington, DC. 97-123.
- Baronti, C., Curini R., D’Ascenzo G., et al. 2000. Monitoring natural and synthetic estrogens at activated sludge sewage treatment plants and in a receiving river water. *Environ Sci Technol*. 34:5059–66
- Bauman, L. and Stenstrom, M. 1990. Removal of organohalogen and organohalogen precursors in reclaimed wastewater. *Water Research*. 24:949-955.
- Beitel, S., Doering, J., Patterson S., Hecker, H. (2015) Assessment of the sensitivity of three North American fish species to disruptors of steroidogenesis using *in vitro* tissue explants. *Aquatic Toxicology*. 152: 273-283
- Benotti, M., Trenholm, R., Vanderford, B., et al. 2009. Pharmaceuticals and Endocrine Disrupting Compounds in U.S. Drinking Water. *Environ. Sci. Technol*. 43:597-603.
- Bjorkblom C., Hogfors E., Salste L., et al. 2009. Estrogenic and androgenic effects of municipal wastewater effluent on reproductive endpoint biomarkers in three-spined stickleback (*Gasterosteus aculeatus*). *Environ. Tox and Chem*. 28: 1063 – 1071.
- Bortone, S., Davis, W., and Bundrick, C. 1989. Morphological and behavioural characters in mosquito fish as potential indication of exposure to kraft mill effluent. *Bullet of the environmental contamination toxicology*. 43: 207-233.
- Campbell C.G., Borglin S.E., Green F.B., Grayson A., Wozel E., Stringfellow W.T. 2006. Biologically directed environmental monitoring, fate, and transport of estrogenic endocrine disrupting compounds in water: A review. *Chemosphere*. 65: 1265 – 1280.
- CCME. 2006. Municipal Wastewater Effluent in Canada: What is municipal wastewater effluent and why is it an issue.
- Cheshenko, K., Pakdel, F., Segner, H., Kah, O., Eggen, R. (2008) Interference of endocrine disrupting chemicals with aromatase CYP19 expression or activity, and consequences for reproduction of teleost fish. *General and Comparative Endocrinology*. 155(1): 31-62
- City of Regina. Water Treatment Plant-Project Overview. Accessed on January 17th, 2014 from <https://www.regina.ca/residents/water-sewer/wastewater-treatment-plant/>



- Desbrow, C., Routledge, E., Brighty, G., et al. 1998. Identification of estrogenic chemicals in STW effluent. I. Chemical fractionation and in vitro biological screening. *Environ Sci Technol.* 32:1549-1558.
- Diniz, M., Peres, I., and Pihan, J. 2005. Comparative study of the estrogenic responses of mirror carp (*Cyprinus carpio*) exposed to treated municipal sewage effluent (Lisbon) during two periods in different seasons. *Sci Total Environ.* 349:129-139.
- Douxflis, J., Robert, M., Frederic, S., Arnaud, B., Delphine, L., Thome, J. (2007) Do sewage treatment plant discharges substantially impair fish reproduction in polluted rivers? *Science of the Total Environment.* 372: 497-514
- Drysdale, D., and Bortone, S. 1989. Laboratory induction of inter-sexuality in the mosquitofish *Gambusia affinis*, using paper mill effluent. *Bullet of environmental contamination and toxicology.* 43: 611-617.
- Environment Canada. 2010. Wastewater pollution. Available from: <http://www.ec.gc.ca/eu-ww/default.asp?lang=En&n=6296BDB0-1>.
- Fitzpatrick, M.S., Van Der Kraak, G., Schreck, C.B., 1986. Profiles of plasma sex steroids and gonadotropin in Coho Salmon, *Oncorhynchus kisutch*, during final maturation. *Gen. Comp. Endocrinol.* 62, 437-451.
- Folmar, L., Denslow, N., Chow, M. et al. 1996. Vitellogenin induction and reduced serum testosterone concentrations in feral male carp (*Cyprinus carpio*) captured near a major metropolitan sewage treatment plant. *Environ. Health Perspec.* 104:1096-1101.
- Fraz S, Lee AH, Wilson JY (2018) Gemfibrozil and carbamazepine decrease steroid production in zebrafish testes (*Danio rerio*). *Aquat Toxicol* 198:1–9. doi: 10.1016/j.aquatox.2018.02.006
- Goksøyr A. (2006). Endocrine Disruptors in the Marine Environment: Mechanisms of Toxicity and their Influence on Reproductive Processes in Fish. *Jour. Tox. Environ. Heal., Pt. A:* 69: 175-184.
- Han S., Choi K., Kim J., et al. 2010. Endocrine disruption and consequences of chronic exposure to ibuprofen in Japanese medaka (*Oryzias latipes*) and freshwater cladocerans *Daphnia magna* and *Moina macrocopa*. *Aquatic Toxicology.* 98: 256-264. doi: 10.1016/j.aquatox.2010.02.013
- Hanson S., Bagatim T., Steeves K., Wiseman S., Hogan N., Hontela A., Jones P., Giesy J. HM Health Status of Fathead Minnow (*Pimephales Promelas*) Populations in a Municipal Waste Water Effluent Dominated Stream in the Canadian Prairies, Wascana Creek, SK, Canada.

- Hanson S., Bagatim T., Steeves K., Wiseman S., Hogan N., Hontela A., Jones P., Giesy J. HM Molecular Biomarkers as Mechanistic Evidence of Municipal Wastewater Effluent Effects on Wild Fathead Minnow (*Pimephales Promelas*) Populations in an Effluent Dominated Stream, Wascana Creek, SK, Canada.
- Harries, J., Sheahan, D., Jobling, S., et al. 1996. A survey of estrogenic activity in United Kingdom inland waters. *Environ. Toxicol. Chem.* 15:1993-2002.
- Harries, J., Runnalls, T., Hill, E., et al. 2000. Development of a Reproductive Performance Test for Endocrine Disrupting Chemicals Using Pair-Breeding Fathead Minnow (*Pimephales promelas*). *Environ. Sci. Technol.* 34: 3003-3011.
- Hashimoto, S., Bessho, H., Hara, A., et al. 2000. Elevated serum vitellogenin levels and gonadal abnormalities in wild male flounder (*Pleuronectes yokohamae*) from Tokyo Bay, Japan. *Marine Environ. Research.* 49:37-53.
- He, Y., Wiseman, S., Wang, N., Perez-Estrada, L., El-Din, M., Martin, J., Giesy, J. (2012) Transcriptional responses of the brain-gonad-liver- axis of fathead minnows exposed to untreated ozone-treated oil sands process-affected water. *Environmental Science and Technology.* 46: 9701-9708
- Hecker, M., Tyler, C., Hoffman, M., Maddix, S., Karbe, L. (2002) Plasma biomarkers in fish provide evidence for endocrine modulation in the Elbe River, Germany. *Environmental Sciences and Technology.* 36(11): 2311-2321
- Hecker, M., Newsted, J.L., Murphy, M.B., Higley, E.B., Jones, P.D., Wu, R., Giesy, J.P., 2006. Human adrenocarcinoma (H295R) cells for rapid in vitro determination of effects on steroidogenesis: hormone production. *Toxicol. Appl. Pharmacol.* 217, 114-124.
- Hecker, M., Giesy, J.P., 2008. Novel trends in endocrine disruptor testing: the H295R steroidogenesis assay for identification of inducers and inhibitors of hormone production. *Anal. Bioanal. Chem.* 390, 287-291.
- Hemming, J., Waller, W., Chow, M., et al. 2001. Assessment of the estrogenicity and toxicity of a domestic wastewater effluent flowing through a constructed wetland system using biomarkers in male fathead minnows (*pimephales promelas* Rafinesque, 1820). *Environ Toxicol.* 20:2268-2275.
- Hogan, N.S., Currie, S., LeBlanc, S., Hewitt, L.M., MacLatchy, D.L., 2010. Modulation of steroidogenesis and estrogen signalling in the estuarine killifish (*Fundulus heteroclitus*) exposed to ethinylestradiol. *Aquat. Toxicol.* 98, 148-156.
- Hoffman, J.L, and Oris, J. T. 2006. Altered gene expression: A mechanism for reproductive toxicity in zebrafish exposed to benzo[a]pyrene. *Aquatic Toxicology.* p 332-340.

- Jensen, K., Korte, J., Kahl, M., Pasha, M., Ankley, G. (2001) Aspects of basic reproductive biology and endocrinology in the fathead minnow (*Pimephales promelas*). *Comparative Biochemistry and Physiology*. 128: 127-141
- Jensen, K., and Ankley, G. 2006. Evaluation of a commercial kit for measuring vitellogenin in the fathead minnow (*Pimephales promelas*). *Ecotoxicology and Environ Safety*. 64: 101-105.
- Jobling, S., Sheahan, D., Osborne, J., et al. 1998. The identification of estrogenic effects in wild fish. R & D Technical Report W119, Environment Agency, Bristol, UK.
- Jones, P., De Coen, W., Tremblay, L., and Giesy, J. 2000. Vitellogenin as a biomarker for environmental estrogens. *Water Science and Technology*. 42: 1-14.
- Kidd, K., Blanchfield, P., Mills, K., et al. 2007. Collapse of a fish population after exposure to a synthetic estrogen. *PNAS*. 104:8807-8901
- Kime, D.E., 1998. Endocrine disruption in fish. Kluwer Academic Publishers, Boston, Dordrecht, London.
- Kinnberg, K., Korsgaard, B, Bjerregaard, P et al., 2000. Effects of nonylphenol and 17 $\beta$ -estradiol on vitellogenin synthesis and testis morphology in male platyfish *Xiphophorus maculatus*. *J. Exp. Biol*. 203:171-181.
- Kirby, M., Allen, Y., Dyer, R., et al. 2004. Surveys of plasma vitellogenin and intersex in male flounder (*platichthys flesus*) as measures of endocrine disruption by estrogenic contamination in United Kingdom estuaries: Temporal trends, 1996 to 2001. *Environ. Toxic. And Chemistry*. 23:748-758.
- Korte, J., Kahl, M., Jensen, K., Pasha, M., Parks, L., LeBlanc, G., Ankley, G. (2000) Fathead minnow vitellogenin: Complementary DNA sequence and messenger RNA and protein expression after 17 $\beta$ -estradiol treatment. *Environmental Toxicology and Chemistry*. 19(4): 972-981
- Kuch, H., Ballschmitter, K. 2001. Determination of endocrine-disrupting phenolic compounds and estrogens in surface and drinking water by HRGS-(NCI)-MS in the pictogram per litre range. *EnvironSci Technol*. 35:3201-3206.
- Kusk, K., Kruger, T., Long, M., Taxvig, C., et al. 2011. Endocrine potency of wastewater: Contents of endocrine disrupting chemicals and effects measured by in vivo and in vitro assays. *Environmental Toxicology and Chemistry*. 30:413-426.
- Lange A, Paull GC, Hamilton PB, et al. 2011. Implications of persistent exposure to treated wastewater effluent for breeding in wild roach (*Rutilus rutilus*) populations. *Environmental Science and Technology*, 45(4):1673-1679

- Lavado, R., Thibaut, R., Raldua, D., et al. 2003. First evidence of endocrine disruption in feral carp from the Ebro River. *Toxicol and Applied Pharmacology*. 196:247-257.
- Lee, W.K., Yang, S.W., 2002. Relationship between ovarian development and serum levels of gonadal steroid hormones, and induction of oocyte maturation and ovulation in the cultured female Korean spotted sea bass *Lateolabrax maculatus* (Jeom-nong-eo). *Aquaculture*. 207, 169-183.
- Lonard D., Tsai, Sy., O'Malley, B. 2004. Selective estrogen receptor modulators 4-hydroxytamoxifen and raloxifene impact the stability and function of SRC-1 and SRC-3 coactivator proteins. *Mol. Cell. Biol*. 24:14-24.
- Matrinovic, D., Blake, L., Durhan, J., et al. 2007. Reproductive toxicity of vinclozolin in the fathead minnow: confirming an anti-androgenic mode of action. *Environ. Toxicol. And Chem*. 27: 478-488.
- McMaster, M.E., Hewitt, L.M., Tetreault, G.R., Janoscik, T., Boyko, C., Peters, L., Parrott, J.L., Van Der Kraak, G.J., Portt, C.B., Kroll, K.J., Denslow, N.D., 2005. Detailed endocrine assessments of wild fish in the northern river basins, Alberta, in comparison to EEM monitored endpoints. *Water Qual. Res. J. Canada*. 40(3), 299-314.
- Miles-Richardson, S., Kramer, V., Fitzgerald, S., et al. 1998. Effects of waterborne exposure of 17 $\beta$ -estradiol on secondary sex characteristics and gonads of fathead minnows (*Pimephales promelas*). *Aquat. Toxicol*.
- Miles-Richardson, S., Pierens, S., Nichols, K., et al. 1999. Effects of waterborne exposure to 4-nonylphenol and nonylphenol ethoxylate on secondary sex characteristics and gonads of fathead minnows (*Pimephales promelas*). *Environ. Res*. 80: 122-137.
- Miller, W. 1998. Molecular biology of steroid hormone synthesis. *Endocr. Rev*. 9:295-318.
- Mills, L., and Chichester, C. 2005. Review of evidence: Are endocrine-disrupting chemicals in the aquatic environment impacting fish populations? *Science of the Total Environment*. 343: 1-34.
- Moyle, P.B., and Cech, J.J. 1988. Reproduction in "Fishes – An introduction to Ichthyology". (P.B. Moyle, Ed.), 2<sup>nd</sup> Ed., pp. 107-131. Prentice Hall, Englewood Cliffs, N.J.
- Nichols, K., Miles-Richarson, S., Snyder, E., Giesy, J. (1999) Effects of exposure to municipal wastewater *in situ* on the reproductive physiology of the fathead minnow (*Pimephales promelas*). *Environmental Toxicology and Chemistry*. 18(9): 2001-2012
- Palace, V. et al. 2002. Induction of vitellogenin and histological effects in wild fathead minnows from a lake experimentally treated with the synthetic estrogen, ethynylestradiol. *Water Quality Research Journal of Canada*. 37:637-650.

- Pawloski, S., Aerle, R., Tyler, C., Braunbeck, T. (2004) Effects of 17 $\alpha$ -ethinylestradiol in a fathead minnow (*Pimephales promelas*) gonadal recrudescence assay. *Ecotoxicology and Environmental Safety*. 57(3): 330-345.
- Purdom, C., Hardiman, P., Bye, V., et al. 1994. Estrogenic effects of effluents from sewage treatment works. *Chem Ecol* 8:275-285.
- Routledge, E., Sheahan, D., Desbrow, C., et al. 1998. Identification of Estrogenic Chemicals in STW Effluent. 2. In Vivo Responses in Trout and Roach. *Environ. Sci. Technol.* 32:1559-1565.
- Sakamoto, K., Nakai, A., Yokoyama, A., et al. 2003. Cytochrome P450 induction and gonadal status alteration in common carp (*Cyprinus carpio*) associated with the discharge of dioxin contaminated effluent to the Hikiji River, Kanagawa Prefecture, Japan. *Chemosphere*. 51:491-500.
- Sanderson, J. 2006. The Steroid Hormone Biosynthesis Pathway as a Target for Endocrine-Disrupting Chemicals. *Toxicological Sciences* 94: 3-21.
- Smith, R.J.F. 1974. Effects of 17 $\alpha$ -methyltestosterone on the dorsal pad and tubercles of fathead minnows (*Pimephales promelas*). *Can. J. Zool.* 52: 1031-1038.
- Smith, R. J. F. 1978. Seasonal changes in the histology of the gonads and dorsal skin of the fathead minnow, *Pimephales promelas*. *Can. J. Zool.* 56, 2103-2109.
- Solé, M., Porte, C., Barceló, D. 2000. Vitellogenin induction and other biochemical responses in carp, *Cyprinus carpio*, after experimental injection with 17 $\alpha$ -ethynylestradiol. *Arch. Environ. Contam. Toxicol.* 38:494-500.
- Steeves K., Bagatim T., Hanson S., Wiseman S., Hogan N., Hontela A., Jones P., Giesy J. HM Assessing Biological Effects of Municipal Wastewater Effluent Using the Fathead Minnow (*Pimephales Promelas*) Reproductive Bioassay.
- Sumpter, J.P. 1998. Xenoendocrine disrupters- environmental impacts. *Toxicology Letters*. 102-103, 337-342.
- Sumpter, J., Jobling, S. (1995) Vitellogenesis as a biomarker for estrogenic contamination of the aquatic environment. *Environmental Health Perspectives*. 103(7): 173-178
- Tabb, M., and Blumberg, B. 2006. New Modes of Action for Endocrine-Disrupting Chemicals. *Molecular Endocrinology*. 20(3):475-482.
- Sumpter, J.P. 1998. Xenoendocrine disrupters- environmental impacts. *Toxicology Letters*. 102-103, 337-342.

- Tetreault, G., Bennett, C. J., Cheng, C., et al., 2012. Reproductive and histopathological effects in wild fish inhabiting an effluent-dominated stream, Wascana Creek, SK, Canada. *Aquat. Toxicol.* 110: 149-161.
- Tilton, F., Benson, W., and Schlenk, D. 2002. Evaluation of estrogenic activity from a municipal wastewater treatment plant with predominantly domestic input. *Aquat. Toxicol.* 61: 211-224.
- US Environmental Protection Agency (2009) Endocrine disruptor screening program test guidelines—OPPTS 890.1350: Fish short-term reproduction assay. EPA 740/C09/007. Washington, DC
- U.S. EPA. 2011. Endocrine disruptor screening program test guidelines. OPPTS 890.1550: steroidogenesis (human cell line – H295R).
- Vajda, A., Barber, L., Gray, J., et al. 2008. Reproductive Disruption in Fish Downstream from an Estrogenic Wastewater Effluent. *Environ. Sci. Technol.* 42:3407-3414.
- Veldhoen N, Skirrow RC, Osachoff H, et al (2006) The bactericidal agent triclosan modulates thyroid hormone-associated gene expression and disrupts postembryonic anuran development. *Aquat Toxicol.* doi: 10.1016/j.aquatox.2006.08.010
- Vidal-Dorsch, D., Colli-Dula, R., Bay, S., Greenstein, D., Wiborg, L., Petschauer, D., Denslow, N. (2013) Gene expression of fathead minnows (*Pimephales promelas*) exposed to two types of treated municipal wastewater effluents. *Environmental Science and Technology*. 47(19): 11268-11277
- Villeneuve, D., Knoebel, I., Kahl, M., et al. 2006. Relationship between brain and ovary aromatase activity and isoform-specific aromatase mRNA expression in the fathead minnow (*Pimephales promelas*). *Aquat. Toxicol.* 2006. 76: 353-368.
- Villeneuve, D., Mueller, N., Martinovic, D., et al. 2009. Direct effects, compensation and recovery in female fathead minnows exposed to a model aromatase inhibitor. *Environ. Health Perspect.* 117(4):624-631.
- Vos, J., Dybing, E., Greim, H., et al. 2000. Health Effects of Endocrine-Disrupting Chemicals on Wildlife, with Special Reference to the European Situation. *Critical Reviews in Toxicology*. 30-71-133.
- Waiser, M., Tumber, V., and Holm, J. 2011. Effluent-Dominated Streams. Part 1: Presence and effects of excess nitrogen and phosphorous in Wascana Creek, Saskatchewan, Canada. *Environ. Toxicol. And Chem.* 30:496-507.

- Waiser, M., Humphries, D., Tumber, V., and Holm, J. 2011. Effluent-Dominated Streams. Part 2: Presence and possible effects of pharmaceuticals and personal care products in Wascana Creek, Saskatchewan, Canada. *Environ. Toxicol. and Chem.* 30:508-519
- Weeks, J., Guiney, P., Nikiforov, A. (2012) Assessment of the environmental fate and ecotoxicity of N,N-diethyl-m-toluamide (DEET). *Integrated Environmental Assessment and Management*. 8(1): 120-134.
- WHO/IPCS. 2002. Global assessment of the state-of-the-science of endocrine disruptors. World Health Organization/International Program on Chemical Safety. WHO/PCS/EDC/02.2. Available at: [www.who.int/pcs/emerg\\_site/edc/global\\_edc\\_ch5.pdf](http://www.who.int/pcs/emerg_site/edc/global_edc_ch5.pdf)
- Zenobio JE, Sanchez BC, Archuleta LC, Sepulveda MS (2014) Effects of triclocarban, N,N-diethyl-meta-toluamide, and a mixture of pharmaceuticals and personal care products on fathead minnows (*Pimephales promelas*). *Environ Toxicol Chem* 33:910–919. doi: 10.1002/etc.2511

## Appendix A: Supplemental Materials

Supplementary materials to be submitted with manuscripts are included here. The figure or table number is presented as Cx.Sy format, where ‘Cx’ indicates chapter number, and ‘Sy’ indicates figure or table number.

**C2.S1.** Summary of primary histological diagnostic criteria in male and female fathead minnows adapted from the Diagnosis of Endocrine-related Histopathology of Fish Gonads (US EPA 2009).

Primary Criteria	Males	Females
1.	Increased proportion of spermatogonia	Increased oocyte atresia
2.	Presence of testicular oocytes	Perifollicular cell hyperplasia
3.	Increased testicular degeneration	Decreased yolk formation
4.	Interstitial cell hyperplasia	Change in gonadal staging



## **Appendix B: Histological Staining**

### **Processing Sequence**

- a. 10% neutral buffered formalin – 1 hour
- b. 10% alcoholic formalin – 1 hour
- c. 90% ethanol – 1 hour
- d. Absolute ethanol – 1 hour
- e. Absolute ethanol – 1 hour
- f. Absolute ethanol – 1 hour
- g. Absolute ethanol – 1 hour
- h. Xylene – 1 hour
- i. Xylene – 1 hour
- j. Xylene – 1 hour
- k. Paraplast wax – 1 hour
- l. Paraplast wax – 1 hour
- m. Paraplast wax – 1 hour
- n. Paraplast wax – 1 hour

### **Hematoxylin and Eosin Staining**

- a. Xylene (1) – 2 min
- b. Xylene (2) – 2 min
- c. 100% Ethanol (1) – 2 min
- d. 100% Ethanol (2) – 2 min
- e. 95% Ethanol – 2 min
- f. 70% Ethanol – 2 min
- g. Dip into running water – 5 sec
- h. Dip into distilled water – 5 sec
- i. Hematoxylin – 2 min
- j. Rinse in running water
- k. Dip into acid alcohol – 5 sec
- l. Running water – 10 min
- m. Dip into distilled water – 5 sec
- n. Eosin – 1 min
- o. 2 dips into running water – 5 sec
- p. Dip into 70%, 95% and 100% Ethanol – 5 sec
- q. 100% Ethanol (2) – 1 min
- r. Xylene – 2 min

## Appendix C: Histological Grading and Staging Scale

### General severity grading scale

*Non Remarkable:* This grade is used if there are no findings associated with a particular diagnostic criterion.

*Grade 1: Minimal.* Ranging from inconspicuous to barely noticeable but so minor, small, or infrequent as to warrant no more than the least assignable grade. For discrete changes, grade 1 is used when there are fewer than 2 occurrences per microscopic field, or 1-2 occurrences per section. For multifocal or diffusely-distributed alterations, this grade is used for processes where  $\leq 20\%$  of the tissue in the section is involved.

*Grade 2: Mild.* A noticeable feature of the tissue. For discrete changes, grade 2 is used when there are 3-5 occurrences per microscopic field or per tissue section. For multifocal or diffusely-distributed alterations, this grade is used for processes where 20-50% of the tissue in the section are involved.

*Grade 3: Moderate.* A dominant feature of the tissue. For discrete changes, grade 3 is used when there are 6-8 occurrences per microscopic field or per tissue section. For multifocal or diffusely-distributed alterations, this grade is used for processes where 50-80% of the tissue in the section are involved.

*Grade 4: Severe.* An overwhelming feature of the tissue. For discrete changes, grade 4 is used when there are more than 9 occurrences per microscopic field or per tissue section. For multifocal or diffusely-distributed alterations, this grade is used for processes where  $>80\%$  of the tissue in the section are involved.

### Criteria for Staging Ovaries

*Juvenile:* gonad consists of oogonia exclusively; it may be difficult or impossible to confirm the sex of these individuals

*Stage 0 – Undeveloped:* entirely immature phases (oogonia to perinucleolar oocytes); no cortical aleoli.

*Stage 1 – Early Development:* vast majority (e.g.,  $>90\%$ ) are pre-vitellogenic follicles, predominantly perinucleolar through cortical alveolar.

*Stage 2 – Mid-Development:* at least half of the observed follicles are early and mid-vitellogenic.

*Stage 3 – Late Development:* majority of developing follicles are late vitellogenic

*Stage 4 – Late Development/hydrated:* majority of follicles are late vitellogenic and mature/spawning follicles; follicles are larger as compared to Stage 3.

*Stage 5 – Post-ovulatory:* predominately spent follicles, remnants of theca externa and granulosa.